(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 375 437 B1

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
09.09.1998 Bulletin 1998/37

(51) Int Cl.[6]: **C07K 14/62**, C12P 21/02, A61K 38/28, C12N 15/17

(21) Application number: **89313460.1**

(22) Date of filing: **21.12.1989**

(54) **Human insulin analogues**

Analoge vom menschlichen Insulin

Analogues de l'insuline humaine

(84) Designated Contracting States:
AT BE CH DE ES FR GB GR IT LI LU NL SE

(30) Priority: **23.12.1988 DK 721588**
28.09.1989 DK 477789

(43) Date of publication of application:
27.06.1990 Bulletin 1990/26

(60) Divisional application: **97121213.9 / 0 837 072**
97121214.7 / 0 861 851

(73) Proprietor: **NOVO NORDISK A/S**
2880 Bagsvaerd (DK)

(72) Inventors:
- **Balschmidt, Per**
DK-3060 Espergaerde (DK)
- **Brange, Jens Jorgen Vellgaard**
DK-2930 Klampenborg (DK)

(74) Representative: **Noergaard, Torsten et al**
c/o Novo Nordisk A/S
Novo Allé
2880 Bagsvaerd (DK)

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EP-A- 0 214 826 **EP-A- 0 254 516**

- **PROTEIN ENGINEERING, vol. 1, no. 3, June 1987, page 238, Eynsham, Oxford, GB, J. BRANGE et al.**

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Printed by Jouve, 75001 PARIS (FR)

Description

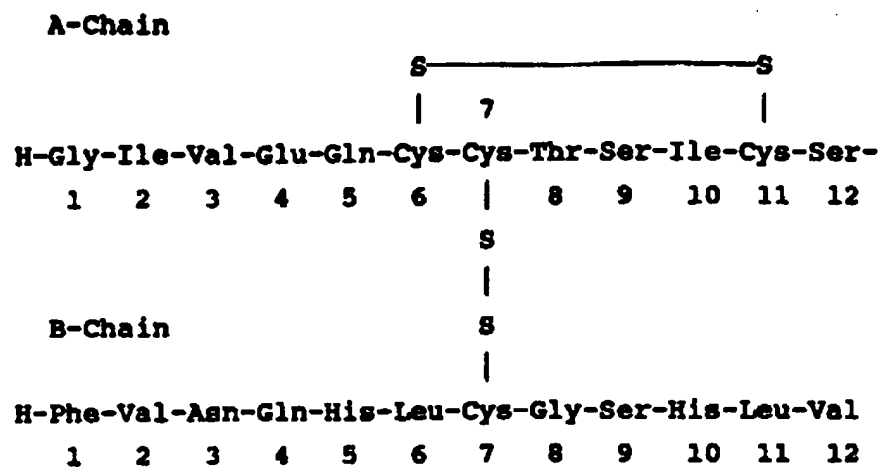
TECHNICAL FIELD

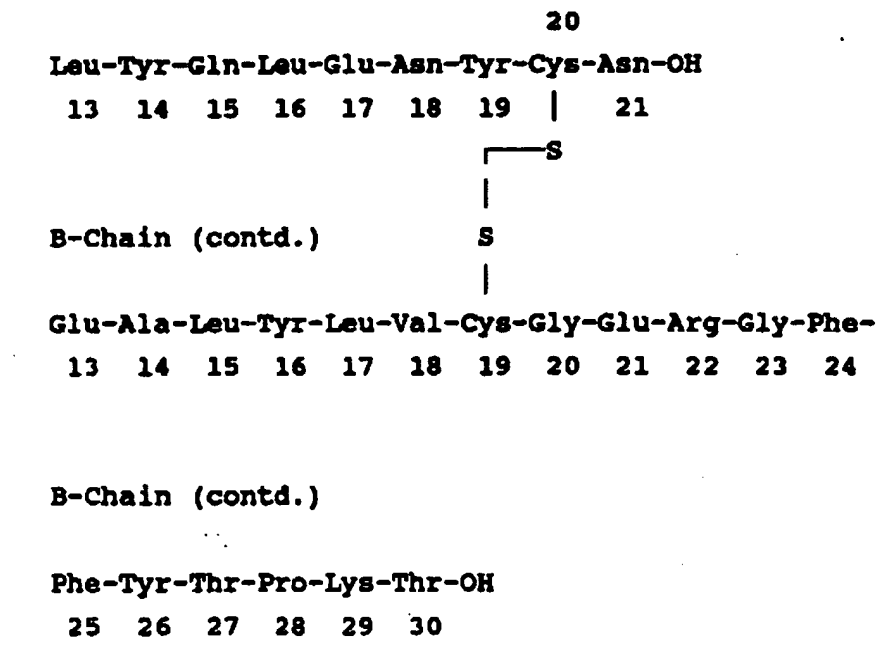
The present invention relates to novel human insulin analogues exhibiting a low ability to associate in solution, a method for the preparation of such insulin analogues, insulin preparations containing the human insulin analogues of the invention and a method of treating Diabetes Mellitus using these human insulin analogues.

BACKGROUND ART

Ever since the discovery of insulin in 1922 many different types of insulin preparations have been used for the treatment of Diabetes mellitus. At the beginning exclusively insulin solutions exhibiting a rapidly commencing and relatively rapidly ceasing insulin activity were used, but later on insulin preparations exhibiting a wider profile of activity procured by lowering the solubility of insulin by means of additions as e.g. zinc salt and/or protamines have been produced. For reasons of availability the insulin used herefor has normally been recovered from Pancreas from domestic animals, most frequently oxes, pigs and sheep, however, recently preparations containing human insulin of biotechnological origin have also appeared on the market.

The structure of human insulin is shown in the following formula





B-Chain (contd.)

Phe-Tyr-Thr-Pro-Lys-Thr-OH
25 26 27 28 29 30

The insulins from certain domestic animals are very similar in structure to human insulin. Thus dog and pig insulin differ from human insulin only by containing Ala in position 30 in the B-chain and rabbit insulin only by containing Ser in the same position. These insulins may be converted into human insulin by replacement of the B30-amino acid residue with Thr by semisynthetic procedures as described by Morihara et al, Nature 280 (1979), 412-413 and Marcussen (US Patent N . 4,343,898).

When such an insulin is dissolved at physiological pH value a concentration-dependent association equilibrium is established between monomeric, dimeric, tetrameric, hexameric and even polymeric insulin. The equilibrium can e.g. be determined by ultracentrifugation, by osmometry or by gel filtration methods, vide e.g. R. Valdes Jr. and G.A. Ackers, "Methods in enzymology", vol. 61 (Enzyme Structure, part H. eds.: Hirs & Timasheff), Academic Press 1979, pages 125-142. In normal formulations of insulin preparations this equilibration is shifted in such a way that the insulin t a very high degree is on a hexameric form.

Substitutions in the insulin molecule can be introduced with the purpose of improving the profile of activity of the insulin in the treatment of Diabetes. Thus, Published International Application No. WO 86/05497 discloses that one or more substitutions of Glu in the insulin molecule by a neutral amino acid residue causes a shifting of the zone of precipitation of the insulin in such a way that a slow release after injection is obtained.

Moreover, Published European Application No. EP 214 826 discloses insulin analogues being particularly rapidly absorbed after injection. This effect is a result of the fact that by means of certain substitutions in particular in the B9-B12 region and in the B26-B28 positions in the insulin molecule a suppression of the association tendency of the insulin is obtained so that it is essentially present as monomer or dimer. However, a number of these insulin analogues exhibits a reduced biological activity.

Throughout the years a large number of artificially prepared analogues of human insulin has been described, usually with the purpose of elucidating the influence of the structure on the activity, vide e.g. Märke et al., Hoppe-Seyler's Z. Physiol. Chem. 360 (1979), 1619-1632. Investigations of the influence of substitutions in the (B22-B26)-sequence of the insulin on the receptor binding have been of particular interest, as said sequence is considered to be an essential site of binding for the insulin receptor, and as naturally occurring mutations have been found with substitutions in said site. Vide e.g. S. Shoelson et al. PNAS 80 (1983), 7390-7394 and M. Kobayashi et al.: Biomed. Res. 5 (3) (1984), 267-272. Very low biological activities were found for analogues in which Phe (B24) or Phe (B25) are substituted, and therefore it was concluded that the presence of these two amino acids is of decisive importance to the receptor binding.

The present invention is based on the surprising recognition that certain human insulin analogues in which one of the amino acid residues [Phe^{B24}] or [Phe^{B25}] is not present exhibit a low association tendency in solution and at the same time exhibits an unchanged or even higher in vitro biological activity than human insulin. The deletion of either [Phe^{B24}] or [Phe^{B25}] will have the effect that [Lys^{B29}] is transferred into [Lys^{B28}]. The position of a positive charge in this position in the human insulin molecule is considered to be the important aspect of the present invention.

SUMMARY OF THE INVENTION

In its broadest aspect the present invention is therefore related to human insulin analogues in which there is a positively charged amino acid residue, i.e. Lys or Arg in the position B28, i.e. in position 8 in the B-chain calculated from [Gly^{B20}].

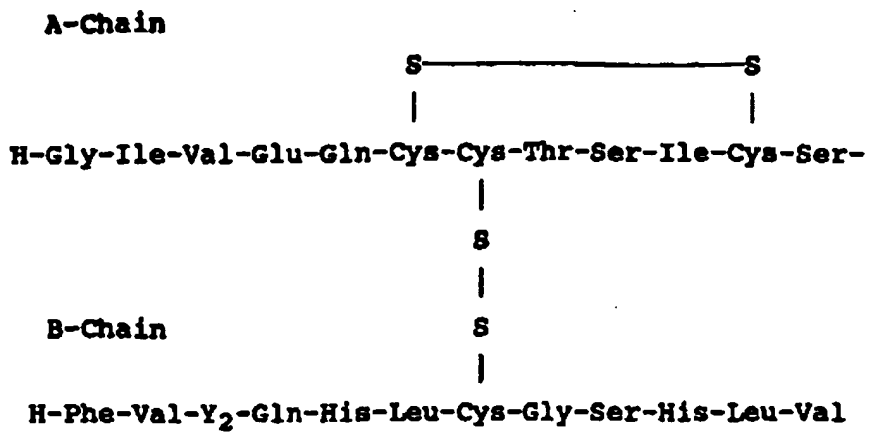
The present insulin analogues have surprisingly a low association tendency and at the same time an increased physical stability compared to other insulin analogues with low association tendency. Introduction of a positive charge in position B28 may be accomplished in two ways. Either by deleting one of the amino acid residues in position B24, B25, B26, B27 or B28 in the human insulin molecule leading to a human insulin analogue with a Lys in position B28 or by substituting [Pro^{B28}] in the human insulin molecule with a Lys or Arg. If an Arg is preferred in position B28 the deletion of one of the amino acid residues in position B24, B25, B26, B27 or B28 may furthermore be combined with a substitution of the original [Lys^{B29}] with an Arg residue.

The present human insulin analogues may furthermore contain one or more modifications in the C-terminal end of the B-chain compared to human insulin. Thus, the amino acid residues in position B25 to B27 and the amino acid residue(s) following the [Lys^{B28}]or or [Arg^{B28}]may be arbitrarily chosen among the naturally occurring amino acid residues with the proviso that there is no Pro in position B29 or B29 or B30 or both may be lacking.

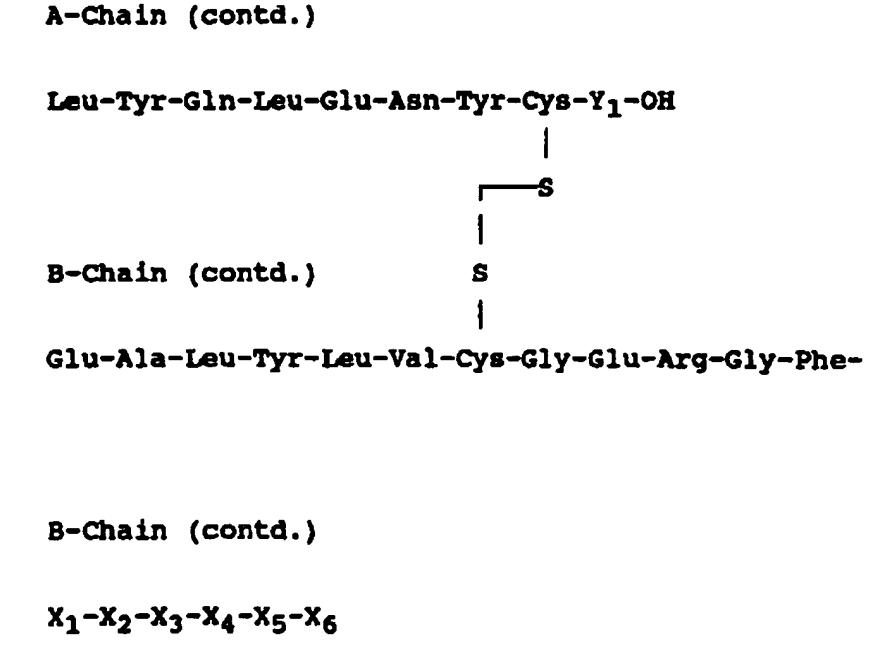
According to one aspect of the present invention [Tyr^{B26}] may be substituted by another uncharged amino acid residue wherein the second carbon atom in the side chain (Cγ) is sp^2-hybridized (the bonds having a planar structure).

Also with the purpose of stabilizing the molecule against chemical degradation, Asn in position A21 and/or B3 may furthermore be replaced by another amino acid residue.

The present human insulin analogues can be characterized by the following formula I



(I)



B-Chain (contd.)

X_1-X_2-X_3-X_4-X_5-X_6

wherein X_1, X_2, X_3, Y_1 and Y_2 may be any naturally occurring amino acid residue, X_4 is Lys or Arg, X_5 is selected from the group consisting of any naturally occurring amino acid residue except Pro, X_6 may be any naturally occurring amino acid residue carrying the C-terminal hydroxy group or -OH or X_5 and X_6 together form the C-terminal hydroxy group.

In the above formula Y_1 and/or Y_2 may in one embodiment be selected from the group consisting of any naturally occurring amino acid residue except Asn.

In the above formula I X_1 may more specifically be Phe, Ala, His, Thr, Ser, Asn or Tyr,

X_2 may more specifically be Tyr, Thr, Glu, Asp, Ala, His, Ser or Phe,

X_3 may more specifically be Pro, Glu, Asp, Ser, Thr or His,

X_5 may more specifically be Lys, Thr, Ser, Ala, Asp or Glu,

X_6 may more specifically be Thr-OH, Ser-OH, Ala-OH, Asp-OH, Glu-OH or -OH,

Y_1 may be Asn, Glu, Asp, His, Ser, Thr, Val, Leu, Ile, Ala, Met, Trp, Tyr, Gln or Gly, more preferably Gly, Asp, Glu or Ala, and

Y_2 may be Asn, Glu, Asp, His, Ser, Thr, Val, Leu, Ile, Ala, Met, Trp, Tyr, Gln or Gly, more preferably Glu or Asp.

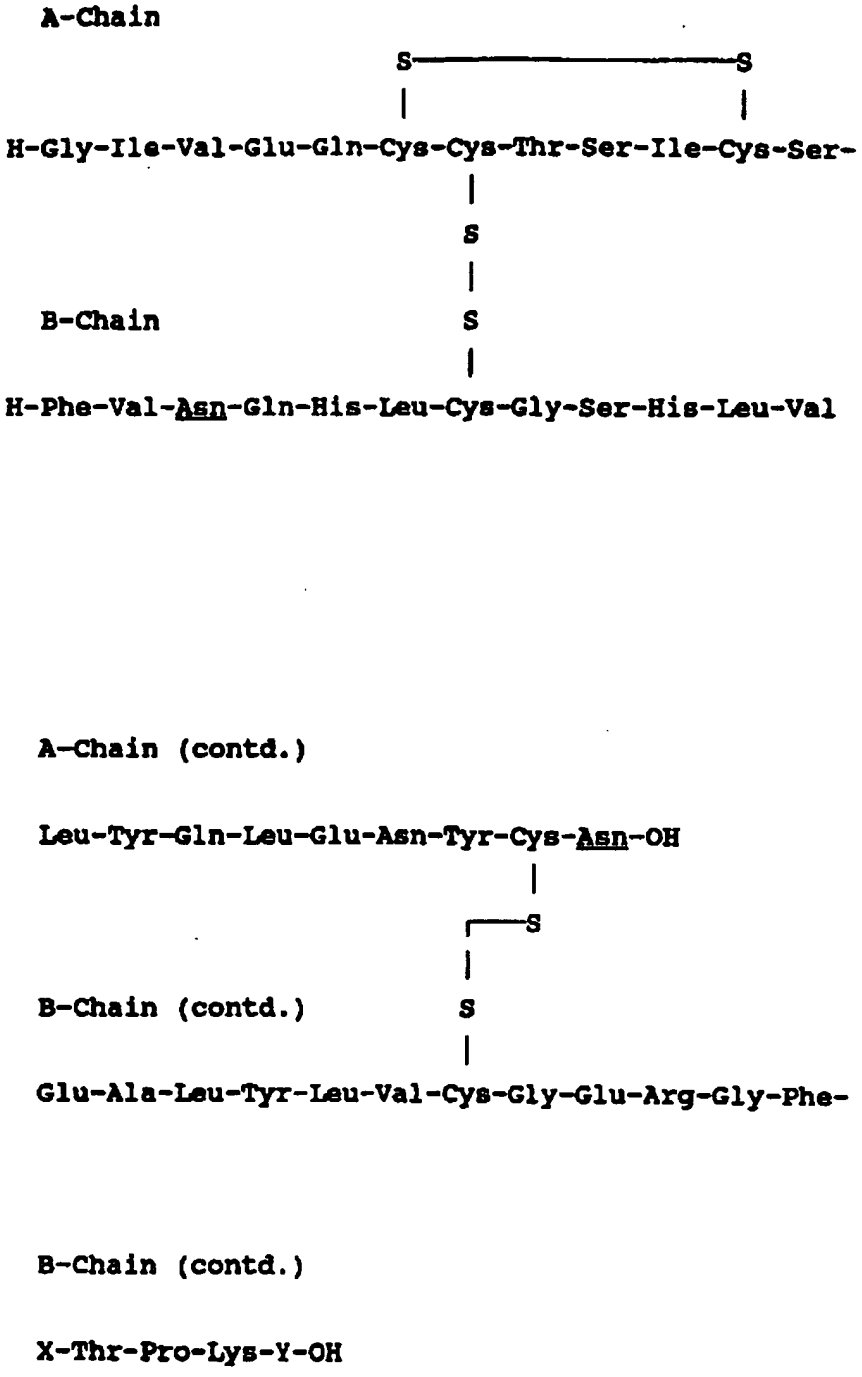
One group of the present human insulin analogues can be characterized as such in which one of the amino acid residues in position B24 or B25 has been deleted, that the amino acid residue in position B26, optionally, is substituted by another uncharged amino acid residue in which the carbon atom in the γ-position is sp^2-hybridized, that, optionally, one or more of the amino acid residues in positions A21, B3 and B30 differ from the amino acid residue in the corresponding positions in human insulin, and that, optionally, no amino acid residue is present in position B30.

According to a more simple definition such analogues are human insulin analogues in which [Tyr^{B26}] is not present, in which [Phe^{B25}] has optionally been substituted by another uncharged amino acid residue in which the carbon atom in the γ-position is sp^2-hybridized, in which one or more of the amino acid residues in positions A21, B3 and B30, optionally, differ from the amino acid residues in human insulin and in which optionally no amino acid residue is present in position B30.

Examples of uncharged amino acid residues in which α_γ is sp^2-hybridized are Tyr, Phe, His, Trp and Asn.

It is possible to introduce further substitutions or derivatizations in the human insulin analogues mentioned above if the properties do not change substantially. Such further derivatizations could be esterification or amidation of carboxyl groups, acylation or alkylation of amino- or hydroxyl groups or could be deamidation of carboxamide groups. Further substitutions may be exchange of [Thr^{A8}] with His or of [His^{B10}] with Asp. Moreover, it is possible to add or delete a single or a few amino acid residues at the C- and/or the N-terminal of preferably the B-chain.

One group of the human insulin analogues according to the invention will have the structure shown in formula II below, where X means Tyr, His, Phe or Asn, Y means Thr, Ser, Ala, Asp or Glu or a deletion and where optionally one or both of the underscored Asn have been changed to Asp by substitution or deamidation or the underscored Asn in the A-chain may be Gly.



(II)

B-Chain (contd.)

X-Thr-Pro-Lys-Y-OH

Preferred human insulin analogues according to the invention are the following:

des[Phe^{B25}]-human insulin
des[Tyr^{B26}]-human insulin
des[Thr^{B27}]-human insulin
des[Pro^{B28}]-human insulin

des[Phe^{B25}]-porcine insulin
des[Pro^{B28}]-porcine insulin
des[Pro^{B28}]-rabbit insulin
des[Phe^{B25}],des[Thr^{B30}]-human insulin
des[Tyr^{B26}],des[Thr^{B30}]-human insulin
[Ser^{A21}]-des[Pro^{B28}]-human insulin
[Gly^{A21}]-des[Pro^{B28}]-human insulin
[Gly^{A21}]-des[Phe^{B25}]-human insulin
[Asp^{A21}]-des[Phe^{B25}]-human insulin
[His^{B25}]-des[Tyr^{B26}],des[Thr^{B30}]-human insulin
[Asn^{B25}]-des[Tyr^{B26}],des[Thr^{B30}]-human insulin
[Asp^{A21}]-des[Phe^{B25}],des[Thr^{B30}]-human insulin
[Asp^{B28}]-des[Phe^{B25}]-human insulin
[Asp^{B3}]-des[Phe^{B25}]-human insulin
[Lys^{B28}]-human insulin
[Lys^{B28},Thr^{B29}]-human insulin
[Arg^{B28}]-des[Lys^{B29}]-human insulin

The human insulin analogues according to the present invention may advantageously be used in the treatment of Diabetes as the decreased ability to association leads to a faster uptake in the bloodstream than an ordinary insulin not only after the normally used subcutaneous injection but also by non-parenteral use, vide e.g. Published International Application No. WO87/06137. Also their improved physical stability will make them more advantagous in the Diabetes treatment.

The insulin analogues according to the present invention may be prepared by altering the proinsulin gene through replacement of codon(s) at the appropriate site in the native human proinsulin gene by codon(s) encoding the desired amino acid residue substitute(s) and/or by deleting the codon(s) corresponding to the desired deletion(s). Alternatively, the whole DNA-sequence encoding the desired insulin analogue may be synthesized. The gene encoding the desired insulin analogue is then inserted into a suitable expression vector which when transferred to a suitable host organism, e.g. E. coli, Bacillus or yeast, generates the desired product. The expressed product is then isolated from the cells or the culture broth depending on whether the expressed product is secreted from the cells or not.

The novel insulin analogues may also be prepared by chemical synthesis by methods analogue to the method described by Märki et al. (Hoppe-Seyler's Z. Physiol. Chem., 360 (1979), 1619-1632). They may also be formed from separately in vitro prepared A- and B-chains containing the appropriate amino acid residue substitutions and deletions, whereupon the modified A- and B-chains are linked together by establishing disulphide bridges according to known methods (e.g. Chance et al., In: Rick DH, Gross E (eds) Peptides: Synthesis - Structure - Function. Proceedings of the seventh American peptide symposium, Illinois, pp. 721-728).

The insulin analogues may furthermore be prepared by a method analogue to the method described in EP patent application No. 0163529A, the disclosure of which is incorporated by reference hereinto. By such method an insulin precursor of the human insulin analogue wherein the basic amino acid in position B28 or B29 (if the final product shall have a basic amino acid in this position) is connected to Gly^{A1} by means of either a peptide bond or a peptide chain of varying length is expressed and secreted by yeast with correctly positioned disulphide bridges and is then converted into the desired human insulin analogue by the Morihara method (Morihara supra) or the so-called transpeptidation reaction (see US patent No. 4,343,898).

Accordingly the present insulin analogues may be prepared by inserting a DNA-sequence encoding a precursor of the insulin analogue in question into a suitable yeast expression vehicle which when transferred to yeast is capable of expressing and secreting the precursor of the insulin analogue in which [Lys^{B28}], [Arg^{B28}], [Lys^{B29}] or [Arg^{B29}] is connected to Gly^{A1} by a peptide bond or a peptide chain with the formula III

$$-R_n-R^1- \quad \text{(III)}$$

wherein R is a peptide chain with n amino acid residues, n is an integer from 0 to 33 and R^1 is Lys or Arg when culturing the transformed yeast strain in a suitable nutrient medium. The precursor is then recovered from the culture broth and reacted with an amino compound with the formula IV

$$Q\text{-}OR' \quad \text{(IV)}$$

wherein Q is a single amino acid residue, preferably Thr, or a dipeptide, and R" is a carboxy protecting group (e.g. methyl or tert-butyl), using trypsin or trypsin-like enzyme as a catalyst in a mixture of water and organic solvents analogously as described in US patent specification No. 4,343,898 (the disclosure of which is incorporated by reference hereinto) whereupon the carboxy protecting group is removed and the insulin analogue is isolated from the reaction mixture.

If the insulin analogues contain an amino acid residue different from Lys or Arg as the C-terminal residue in the B-chain, they may also be prepared by a method analogue to the method described in Published European Application No. EP 195 691 the disclosure of which is incorporated by reference hereinto. By this method insulin analogue precursors of the type having a bridge between the A- and B-chain consisting of a single pair of basic amino acid (Lys, Arg) are made in yeast and then converted into the insulin analogue by an enzymatic conversion.

If the C-terminal amino acid residue in the B-chain is Lys or Arg, then the insulin analogues can be prepared from the above biosyntetic precursors by enzymatic cleavage with trypsin.

Human insulin analogues of the invention in which substitutions are only present within the last amino acid residues nearest to the C-terminal of the B-chain may moreover be prepared in a manner known per se from e.g. porcine insulin as described in K. Inoye et al.; JACS 101 (3), (1979), 751-752, whereby the porcine insulin is first split with trypsin to des-(B23-30)-human insulin, whereupon the latter, also enzymatically, is coupled with a synthetic peptide having the desired amino acid sequence.

The present insulin analogues may be used for the preparation of novel insulin preparations with insulin activity to be substituted for human or porcine insulin in the insulin preparations heretofore known to the art. Such novel insulin preparations contain the insulin analogues according to the present invention or a pharmaceutically acceptable salt thereof in aqueous solution or suspension, preferably at neutral pH. The aqueous medium is made isotonic, for example with sodium chloride, sodium acetate or glycerol. Furthermore, the aqueous medium may contain zinc ions, buffers such as acetate and citrate and preservatives such as m-cresol, methylparaben or phenol. The pH value of the preparation is adjusted to the desired value and the insulin preparation is made sterile by sterile filtration.

The present insulin analogues may also be mixed with other insulin analogues having a protracted insulin activity to prepare insulin preparations consisting of a mixture of rapid acting and protracted insulin.

The insulin preparations of this invention can be used similarly to the use of the known insulin preparations.

TERMINOLOGY

The abbreviations used for the amino acids are those stated in J. Biol. Chem. 243 (1968), 3558. The amino acids are in the L configuration. Unless otherwise indicated, the species of insulins stated herein is human.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is further illustrated with reference to the accompanying drawings in which

Fig. 1 shows the expression plasmid pYGABA 14276,
Fig. 2 shows the yeast vector pAB24,
Fig. 3 shows the DNA sequence of the 0.4 kb EcoRI-XbaI fragment from the plasmid pKFN-864, and
Fig. 4 shows the preparation of the expression plasmid pKFN-866.

DETAILED DESCRIPTION

DNA-sequences encoding modified insulin precursors were constructed with basis in the expression cassette, which is contained in the BamHI restriction fragment from the expression plasmid pYGABA as shown in Figure 1, has a lenght of 1103 basepairs and contains essentially the following (listed in succession starting from the 5'-end): The GAPDH promoter (Travis et al., J. Biol. Chem., 260 (1985), 4384-4389) followed by the coding region consisting of: The 83 N-terminal amino acids of the MF α1-leader sequence encoded by the wild-type yeast DNA-sequence as described by Kurjan & Herskowitz followed by the two codons AAA and AGA encoding Lys and Arg and again followed by the coding region for the insulin precursor single chain des [Thr^{B30}]- human insulin (SCI), which is a synthetically constructed gene using preferred yeast codons. After two stop-codons, a SalI restriction site is positioned, and the rest of the sequence constitutes the Mfα1-sequence containing the terminator region. The sequence is constructed using entirely standard techniques.

The method employed was "oligonucleotide site directed mutagenesis", which is described by Zoller & Smith, DNA, Vol. 3, No. 6 (1984), 479-488. The method is briefly described in the following, and is described thoroughly in Example 1. The insulin precursor sequence is isolated from the expression plasmid and inserted into a single-stranded genom, circular M13 bacteriophage vector. A chemically synthesized complementary DNA-strand is then annealed to the sin-

gle-stranded genom. The DNA-strand contains the desired sequence surrounded by sequences completely homologous to insulin sequences on the circular DNA. The primer is then extended in vitro into the entire length of the circular genom biochemically using Klenow polymerase. This strand will give rise to single-stranded phages, which when grown in E. coli give the possibility of isolating double-stranded DNA with the desired sequence. From this double-stranded DNA, a restriction fragment can be isolated and reinserted into the expression vector.

MODES FOR CARRYING OUT THE INVENTION

The invention is further illustrated by the following Examples.

EXAMPLE I

Construction of an expression plasmid, which can be used to express des[Phe^{B25}]-SCI.

The expression cassette, which is contained in the expression plasmid pYGABA (shown in Figure 1) on a BamHI restriction fragment, was isolated: The expression plasmid was incubated with the restriction endonuclease BamHI. The conditions were: 20 µg of plasmid, 50 units of BamHI, 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT in a volume of 100 µlitres. The temperature was 37°C and the reaction time 2 hours. The two DNA-fragments were separated on a 1% agarose gel, and the desired fragment was isolated.

Ligation to the M13 vector M13mp18:

The isolated restriction fragment was ligated to the bacteriophage vector M13mp18 also cut with the restriction endonuclease BamHI in the following reaction mixture: Fragment 0.2 µg, vector 0.02 µg, 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP in a volume of 20 µlitres. 5 µlitres of this mixture were transformed into the E. coli strain JM101. The presence of fragment in the vector and the orientation of the fragment was determined by restriction enzyme mapping on double-stranded M13-DNA isolated from the transformants.

Isolation of single-stranded (ss) DNA (template):

From the transformant described above ss-DNA was isolated according to a method described by Messing in Gene, 19 (1982), 269-276.

5'phosphorylation of the mutagenisation primer:

The mutagenisation primer with the sequence 5'-TTGGAGTGTAGAAACCTCTT-3' was phosphorylated in the 5'-end in a 30 µlitres reaction mixture containing 70 mM Tris-HCl, pH 7.0, 10 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 100 pmol oligonucleotide and 3.6 units of T4 polynucleotide kinase. The reaction was carried out for 30 min. at 37°C. Then, the enzyme was inactivated by incubating the mixture for 10 min. at 65°C.

Annealing of template and phosphorylated mutagenisation primer:

Annealing of template and primer was carried out in a 10 µlitres volume containing 0.5 pmol template, 5 pmol primer, 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM DTT by heating for 10 min. at 65°C and cooling afterwards to 0°C.

Extension/ligation reaction:

To the reaction mixture above, 10 µlitres of the following mixture were added: 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP, 0.3 mM dTTP 1 mM ATP, 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 3 units of T4 DNA ligase and 2.5 units of Klenow polymerase. Then, the reaction was carried out for 16 hours at 16°C.

Transformation of Jm101:

The reaction mixture above was transformed in different dilutions into $CaCl_2$-treated E. coli JM101 cells using standard techniques and plated in 2 x YT topagar on 2 x YT agar plates. (2 x YT = tryptone 16 g/litre, yeast extract 10 g/litre, NaCl 5 g/litre. 2 x YT topagar = 2 x YT with 0.4% agarose added and autoclaved. 2 x YT agar plates = 2 x YT with 2% agar added and autoclaved). The plates were incubated at 37°C overnight.

Identification of positive clones:

The method used was plaque-lift hybridisation which is described in the following: a nitrocellulose-filter was placed on a plate with a suitable plaque-d nsity, so that the filter was wetted. The filter was then bathed in the following solutions: 1.5 M NaCl, 0.5 M NaOH f r 30 sec., 1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0 for 1 min., 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate) till later use. The filter was dried on 3MM filter paper and baked for 2 hours at 80°C in a vacuum oven.

The mutagenisation primer with the sequence 5'TTGGAGTGTAGAAACCTCTT-3' was labelled radioactively in the 5' end in a 30 µlitres volume containing 70 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 10 pmol oligonucleotide, 20 pmol γ-^{32}P-ATP and 3.5 units of T4 polynucleotide kinase. The mixture was incubated at 37°C for 30 min. and then for 5 min. at 100°C.

The dried filter was prehybridised for 2 hours at 65°C in 6 x SSC, 0.2% bovine-serum albumin, 0.2% Ficoll, 0.2% polyvinylpyrrolidon, 0.2% sodium-dodecyl-sulphate (SDS) and 50 µg/ml salmon-sperm DNA. Then, the reaction mixture containing the labelled probe was added to 15 ml of fresh prehybridisation mix, and the filter was bathed herein overnight at 28°C with gentle shaking. After hybridisation the filter was washed 3 times for each 15 min. in 2 x SSC + 0.1% SDS and autoradiographed. After wash in the same solution, but now at 52°C, and another autoradiography, plaques containing DNA-sequences complementary to the mutagenisation primer were identified.

Re-screening of positive clones:

Because the identified clone is a result of a heteroduplex, the plaque was plated again. The hybridisation and identification were repeated.

Purification of double-stranded M13-phage DNA:

A re-screened clone was used for infection of the E. coli strain JM101. A culture containing approximately 10^8 phages and 5 colonies of JM101 was grown for 5 hours in a 5 ml 2 x YT medium at 37°C. Then, double-stranded, circular DNA was purified from the pellet according to a method described by Birnboim & Doly, Nucleic Acids Res., 2 (1979), 1513.

Isolation of a restriction fragment containing modified insulin precursor:

The DNA-preparation (appr. 5 µg) isolated above was digested with 10 units of the restriction endonuclease BamHI in 60 µlitres of 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT for 2 hours at 37°C. The DNA-products were separated on an agarose-gel, and the fragment was purified from the gel.

Ligation to the yeast vector pAB24 (Figure 2):

The isolated restriction fragment was ligated to the yeast vector pAB24 digested with the restriction endonuclease BamHI in the following reaction mixture: Fragment 0.2 µg, vector 0.02 µg, 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP in a total volume of 20 µlitres. 5 µlitres of this reaction mix was used for transformation of the E. coli strain MC1061, in which the modified expression plasmid was identified and propagated. The plasmid was identical to pYGABA, except for the deleted codon.

Transformation of yeast:

Transformation of the expression plasmid into the yeast strain Saccharomyces cerevisiae JC482ΔpepΔLeu2cir^o (α, his4, pep4, ura3, leu2, cir^o) was carried out as described by Ito et al., J. Bact., Vol. 153, No. 1, (1983), 163-168. The transformed cells were plated on SC-ura medium (0.7% Yeast Nitrogen Base, 2.0% glucose, 0.5% casamino acids, 2.0% agar) for selection for plasmid-containing cells.

EXAMPLE II

Construction of an expression plasmid, which can be used for production of des[Tyr^{B26}]-SCI.

The procedure used was essentially the same as described in example I, except that the mutagenisation primer had the sequence 5'-ACCCTTTGGAGTGAAGAAACCTCT-3', that the hybridization temperature was 36°C, and that the washing temperature after hybridization was 60°C. The modified plasmid has a sequence identical to pYGABA, except for the deleted codon.

EXAMPLE III

Construction of an expression plasmid, which can be used for production of [His^{B25}],des[Tyr^{B26}]-SCI.

The procedure used was essentially the same as described in example I, except that the mutagenisation primer had the sequence 5'-AATACCCTTTGGAGTGTGGAAACCTCTTTCACC-3', that the hybridization temperature was 43°C, and that the washing temperature after hybridization was 66°C. The modified plasmid has a sequence identical to pYGABA, except for the modified and deleted codons.

EXAMPLE IV

Construction of an expression plasmid, which can be used for production of [Asn^{B25}],des[Tyr^{B26}]-SCI.

The procedure used was essentially the same as described in example I, except that the mutagenisation primer had the sequence 5'-AATACCCTTTGGAGTGTTGAAACCTCTTTCACC-3', that the hybridization temperature was 42°C, and that the washing temperature after hybridization was 65°C. The modified plasmid has a sequence identical to pYGABA, except for the modified and deleted codons.

EXAMPLE V

Expression of precursor and isolation from the culture medium.

Yeast, transformed as described in examples I to IV, was propagated on Petri-plates containing minimal-medium without uracil for 48 hours at 30°C. 100 ml shake bottles containing minimal-medium without uracil + 5 g/litre casamino acids + 10 g/litre succinic acid + 30 g/litre glucose at pH 5.0 were inoculated with a single colony from the Petri-plate. The bottles were then shaken at 30°C in incubator for 72 hours.

After centrifugation 1 litre of pooled supernatant was sterilized by filtration and adjusted to pH 4 - 4.5 and a conductivity < 10 mS by addition of 5 M HCl and water. With a flow of 120 ml/hour the supernatant was then applied to a 1.6 x 6 cm column of S/Sepharose®FF previously equilibrated with 50 mM acetic acid, 50% (by volume) ethanol adjusted to pH 4.0 with NaOH. The column was washed with 60 ml buffer and the precursor was then eluted by a linear gradient of NaCl from 0 to 0.35 M in 360 ml buffer with a flow of 10 ml/hour. The eluate was divided in fractions of 4 ml and detected for UV-absorbance. Fractions containing precursor were identified by RP-HPLC analysis and were pooled. After desalting on a column of Sephadex®G25 in 1 M acetic acid the precursor was isolated by lyophilization.

EXAMPLE VI

Preparation of des[Phe^{B25}],des[Thr^{B30}]-human insulin.

400 mg of des[Phe^{B25}]-SCI, prepared by the methods described in examples I and V, were dissolved in 40 ml of 50 mM tris-(hydroxymethyl)aminomethane, 20% (by volume) ethanol adjusted to pH 9 with HCl and 40 ml (settled volume) of Sepharose® containing 32 mg of immobilized trypsin in the same buffer were added. The suspension was left for 24 hours at 8-10°C with gentle agitation and was then filtered. The gel was washed with 40 ml of buffer, and the pooled filtrates were applied to a 2.6 x 7.5 cm column of Q-Sepharose®FF previously equilibrated with 50 mM tris-(hydroxymethyl)aminomethane, 50% (by volume) ethanol, adjusted to pH 8.0 with HCl. The column was then eluted with a linear gradient of NaCl from 0 to 0.15 M in the same buffer over 6 hours with a flow of 225 ml/hour. The eluate was detected for UV-absorbance and fractions containing the main protein peak were pooled. The protein was precipitated at pH 5.4 after removal of the ethanol in vacuo.

250 mg of des[Phe^{B25}],des[Thr^{B30}]-human insulin were isolated by lyophilization.

The identity of the product was confirmed by amino acid analysis, by plasma desorption mass spectrometry and by sequential Edman degradation of the separated vinylpyridylated A-and B-chains.

EXAMPLE VII

Preparation of des[Phe^{B25}]-human insulin.

200 mg of des[Phe^{B25}],des[Thr^{B30}]-human insulin prepared by the methods described in example VI were dissolved in a mixture containing 400 mg of threonine methyl ester, 2.0 ml of ethanol and 0.8 ml of water. The pH value was adjusted to 6.3 with acetic acid and 4 ml (settled volume) of Sepharose® containing 3.2 mg of immobilized trypsin were added. After standing for 2 hours at 20°C with gentle agitation, the gel was removed by filtration, and the protein was precipitated by addition of 10 volumes of 2-propanol. The air-dried precipitate was redissolved in 20 mM tris (hydroxymethyl)aminomethane/HCl, 60% (by volume) ethanol, pH 8.25, and applied to a 2.6 x 20 cm Q-Sepharose®FF column, previously equilibrated with the said buffer, and eluted with a linear NaCl-gradient in the same buffer increasing from 0 to 0.1 M over 15 hours at a flow rate of 125 ml/hour. The ethanol was removed in vacuo from the pooled fractions

containing des [Phe^{B25}]-human insulin-(B30-methyl ester), and the protein was precipitated at pH 6.1. The suspension was centrifugated and the precipitate was lyophilized. The methyl ester was then hydrolyzed for 10 min. in cold 0.1 M NaOH at a protein concentration of 10 mg/ml. The reaction was stopped by adjusting the pH value to 8.5, and 2 volumes of 20 mM tris(hydroxymethyl)-aminomethane/HCl, pH 8.5, were added. The solution was then applied to a 2.6 x 20 cm Q-Sepharose®FF column and eluted as described above. The protein was precipitated at pH 5.5 after removal of the ethanol in vacuo.

80 mg of des[Phe^{B25}]-human insulin were obtained after lyophilization.

The identity of the product was confirmed by amino acid analysis, by plasma desorption mass spectrometry and by sequential Edman degradation of the separated vinylpyridylated A-and B-chains.

EXAMPLE VIII

Preparation of des [Tyr^{B26}],des[Thr^{B30}]-human insulin.

250 mg of des[Tyr^{B26}]-SCI, prepared by the methods described in the examples II and V, were dissolved in 25 ml of 50 mM tris(hydroxymethyl)aminomethane, 20% (by volume) ethanol adjusted to pH 9 with HCl and 25 ml (settled volume) of Sepharose® containing 20 mg of immobilized trypsin in the same buffer were added. The suspension was left for 24 hours at 8-10°C with gentle agitation and was then filtered. The gel was washed with 25 ml of buffer, and the pooled filtrates were applied to a 2.6 x 7.5 cm column of Q-Sepharose®FF previously equilibrated with 50 mM tris-(hydroxymethyl)aminomethane, 50% (by volume) ethanol, adjusted to pH 8.0 with HCl. The column was then eluted with a linear gradient of NaCl from 0 to 0.15 M in the same buffer over 6 hours with a flow of 225 ml/hour. The eluate was detected for UV-absorbance and fractions containing the main protein peak were pooled. The protein was precipitated at pH 5.4 after removal of the ethanol in vacuo.

130 mg of des[Tyr^{B26}],des[Thr^{B30}]-human insulin were isolated by lyophilization.

The identity of the product was confirmed by amino acid analysis and by sequential Edman degradation of the separated vinylpyridylated A- and B-chains.

EXAMPLE IX

Preparation of [His^{B25}],des[Tyr^{B26}],des[Thr^{B30}]-human insulin.

450 mg of [His^{B25}],des[Tyr^{B26}]-SCI, prepared by the methods described in the examples III and V, were dissolved in 45 ml of 50 mM tris-(hydroxymethyl)aminomethane, 20% (by volume) ethanol adjusted to pH 9 with HCl and 45 ml (settled volume) of Sepharose® containing 36 mg of immobilized trypsin in the same buffer were added. The suspension was left for 24 hours at 8-10°C with gentle agitation and was then filtered. The gel was washed with 40 ml of buffer, and the pooled filtrates were applied to a 2.6 x 7.5 cm column of Q-Sepharose®FF previously equilibrated with 50 mM tris(hydroxymethyl)aminomethane, 50% (by volume) ethanol, adjusted to pH 8.0 with HCl. The column was then eluted with a linear gradient of NaCl from 0 to 0.15 M in the same buffer over 6 hours with a flow of 225 ml/hour. The eluate was detected for UV-absorbance and fractions containing the main protein peak were pooled. The protein was precipitated at pH 5.4 after removal of the ethanol in vacuo.

200 mg of [His^{B25}],des[Tyr^{B26}],des[Thr^{B30}]-human insulin were isolated by lyophilization.

The identity of the product was confirmed by amino acid analysis and by sequential Edman degradation of the separated vinylpyridylated A- and B-chains.

EXAMPLE X

Preparation of [Asn^{B25}],des[Tyr^{B26}],des [Thr^{B30}]-human insulin.

150 mg of [Asn^{B25}],des[Tyr^{B26}]-SCI, prepared by the methods described in the examples IV and V, were dissolved in 15 ml of 50 mM tris- (hydroxymethyl)aminomethane, 20% (by volume) ethanol adjusted to pH 9 with HCl and 15 ml (settled volume) of Sepharose® containing 12 mg of immobilized trypsin in the same buffer were added. The suspension was left for 24 hours at 8-10°C with gentle agitation and was then filtered. The gel was washed with 40 ml of buffer, and the pooled filtrates were applied to a 1.6 x 10 cm column of Q-Sepharose®FF previously equilibrated with 50 mM tris(hydroxymethyl)aminomethane, 50% (by volume) ethanol, adjusted to pH 8.0 with HCl. The column was then eluted with a linear gradient of NaCl from 0 to 0.15 in the same buffer over 6 hours with a flow of 90 ml/hours. The eluate was detected for UV-absorbance and fractions containing the main protein peak were pooled. The protein was precipitated at pH 5.4 after removal of the ethanol in vacuo.

80 mg of [Asn^{B25}][Tyr^{B26}],des[Thr^{B30}]-human insulin were isolated by lyophilization.

The identity of the product was confirmed by amino acid analysis and by sequential Edman degradation of the separated vinylpyridylated A- and B-chains.

EXAMPLE XI

Preparation of [Asp^{A21}],des[Phe^{B25}],des[Thr^{B30}]-human insulin.

50 mg f des[Phe^{B26}],des[Thr^{B30}]-human insulin prepared by th methods described in xample VI were dissolved in 10 ml water by adjusting the pH value to 2 with 1 M HCl. The solution was then left for 16 days at 30°C. After cooling (to 20°C) 7.5 g of urea were added and the pH value was adjusted to 8.1 with 1 M NaOH. The solution was then applied to a 1.6 x 20 cm Q-Sepharose®FF column, previously equilibrated with 20 mM tris(hydroxymethyl)-aminomethane/HCl, 7 M urea, pH 8.1 at 4°C, and eluted with a linear NaCl-gradient in the same buffer increasing from 0 to 0.05 M over 24 hours at a flow rate of 40 ml/hour. The pooled fractions containing the protein from the last eluting peak were desalted on a column of Sephadex®G25 in 1 M acetic acid and lyophilized.

30 mg of [Asp^{A21}],des[Phe^{B25}],des[Thr^{B30}]-human [Thr^{B30}]-human insulin were obtained.

The identity of the product was confirmed by amino acid analysis, by 5-step Edman degradation and by C-terminal analysis using carboxypeptidase A.

EXAMPLE XII

Preparation of [Ser^{A21}],des[Pro^{B28}]-human]-human insulin.

Construction of an expression plasmid which can be used for production of [Ser^{A21}],des[Pro^{B28}]-human insulin and preparation of [Ser^{A21}],des[Pro^{B28}]-human insulin.

A pUC-19 derived plasmid, pKFN-864, encoding this analogue was constructed by gapped duplex mutagenesis (Y. Morinaga et al., Biotechnology 2 (1984), 636-639) of plasmid pKFN-734 using the two mutagenic primers NOR-648 CTAGAGCCTGCGGGCTGCGTCTAGCTGCAGTAG and Nor-745 ATTGTTCGACAATACCCTTAGCAGCCTT-GGTGTAGAAGAAACCTCTTTCACC. Plasmid pKFN-734 was constructed by ligating the 0.4 kb EcoRI-XbaI fragment encoding a synthetic yeast signal-leader fused in-frame to a synthetic insulin precursor gene B(1-29) -AlaAlaLys-A (1-21) from plasmid pLaC212spx3 to the 2.7 kb EcoRI-XbaI fragment from pUC-19 (C. Yannisch-Perron et al., Gene 33 (1985), 103-119).

Plasmid pLaC212spx3 is described in Example 3 and in Fig. 6 and 13 of International Patent Application Publication No. WO 89/02463.

The DNA sequence of the 0.4 kb EcoRI-XbaI fragment from pKFN-864 encoding signal-leader-insulin B(1-29, des28 Pro)-AlaAlaLys-A(1-21, 21 Ser) is given in Fig. 3.

pKFN-864 was cut with EcoRI and XbaI and the 0.5 kb fragment was ligated to the 9.5 kb NcoI-XbaI fragment from pMT636 and the 1.4 kb NcoI-EcoRI fragment from pMT636, resulting in plasmid pKFN-866, see fig. 4. Plasmid pMT636 was constructed from pMT608 after deletion of the LEU-2 gene and from pMT479, see fig. 4. pMT608 is described in EP 195 691. pMT479 is described in EP 163 529. pMT479 contains the Schizosaccharomyces pombe TPI gene (POT), the S. cerevisiae triosephosphate isomerase promoter and terminator, TPI_P and TPI_T (Alber, T. and Kawasaki, G. J. Mol. Appl. Gen. 1 (1982), 419-434). Plasmid pKFN-866 contains the following sequence: TPI_P-signal-leader-insulin B(1-29, des28 Pro)-AlaAlaLys-A(1-21, 21 Ser) -TPI_T.

S. cerevisiae strain MT663 (E2-7B XE11-36 a/α, ΔtpiΔtpi, pep 4-3/pep 4-3) was grown on YPGaL (1% Bacto yeast extract, 2% Bacto peptone, 2% galactose, 1% lactate) to an O.D. at 600 nm of 0.6.

100 ml of the resulting culture was harvested by centrifugation, washed with 10 ml of water, recentrifuged and resuspended in 10 ml of a solution containing 1.2 M sorbitol, 25 mM Na_2EDTA pH = 8.0, and 6.7 mg/ml dithiotreitol. The suspension was incubated at 30°C for 15 minutes, centrifuged and the cells resuspended in 10 ml of a solution containing 1.2 M sorbitol, 10 mM Na_2EDTA, 0.1 M sodium citrate, pH = 5.8, and 2 mg Novozym® 234. The suspension was incubated at 30°C for 30 minutes, the cells collected by centrifugation, washed in 10 ml of 1.2 M sorbitol and 10 ml of CAS (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris HCL (Tris = Tris(hydroxymethyl)amino methane) pH = 7.5) and resuspended in 2 ml of CAS. For transformation 0.1 ml of CAS-resuspended cells were mixed with approximately 1 µg of plasmid pKFN-866 and left at room temperature for 15 minutes. 1 ml of (20% polyethylenglycol 4000, 10 mM $CaCl_2$, 10 mM Tris HCl, pH = 7.5) was added and the mixture left for further 30 minutes at room temperature. The mixture was centrifuged and the pellet resuspended in 0.1 ml of SOS (1.2 M sorbitol, 33% v/v YPD, 6.7 mM $CaCl_2$, 14 µg/ml leucine) and incubated at 30°C for 2 hours. The suspension was then centrifuged and the pellet resuspended in 0.5 ml of 1.2 M sorbitol. Then, 6 ml of top agar (the SC medium of Sherman et al., (Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) containing 1.2 M sorbitol plus 2.5% agar) at 52°C was added and the suspension poured on top of plates containing the same agar-solidified, sorbitol containing medium. Transformant colonies were picked after 3 days at 30°C, reisolated and used to start liquid cultures. One such transformant KFN-883 was selected for further characterization.

Yeast strain KFN-883 was grown on YPD medium (1% yeast extract, 2% peptone (from Difco Laboratories), and 2% glucose). A 10 ml culture of the strain was shaken at 30°C to an O.D. at 600 nm of 20. After centrifugation the supernatant was analyzed by HPLC as described (L. Snel et al., Chromatographia 24 (1987), 329-332). The yield was

about 0.14 mg/liter of insulin B(1-29, des 28 Pro)-AlaAlaLys-A(1-21, 21 Ser).

The single chain insulin precursor was isolated from the fermentation supernatant by adsorption to an ion exchange column at low pH, desorption at high pH and precipitation of the pool by zinc ions. Transpeptidation of the precursor to [Ser^{A21}],des[Pro^{B28}],[Thr^{B30}-OM]-human insulin was as follows:

10 mmol (2.35 g) of threonine methylester and glacial acetic acid was dissolved in DMF to give 5 ml, 2.5 ml 76.5% v/v DMF in water was added and 0.5 g of precursor was dissolved in the mixture, which was kept at 12°C; then 50 mg of trypsin in 1.25 ml 0.05 M calcium acetate was added and after 24 hours at 12°C the reaction mixture was added to 100 ml of acetone for precipitation of the peptides, which were spun down and dried in vacuo.

The isolated insulin analogue ester was purified on a preparative HPLC column using a silica-C18 matrix at acidic pH. Then the purified ester was hydrolyzed in aqueous medium at pH 10 and 25°C for 24 hours. The [Ser^{A21}],des [Pro^{B28}]-human insulin formed was precipitated at neutral pH with zinc ions. The precipitate was purified by anion exchange chromatography and subsequently desalted by gel filtration. Yield of lyophilized [Ser^{A21}],des[Pro^{B28}]-human insulin was 102 mg.

EXAMPLE XIII

Preparation of des[Thr^{B27}]-human insulin.

1 g of Zn-free porcine insulin was dissolved in 40 ml of water by adjusting the pH value to 9, and a solution of 50 mg of porcine trypsin in 10 ml of 0.25 M ammonium hydrogen carbonate adjusted to pH 9 with ammonia solution was added. The solution was then left at 4°C and after 48 hours a yield of 65% was found by HPLC analysis. The reaction mixture was then gel filtrated at 4°C on a 5 x 90 cm column of Sephadex® G50 superfine in 0.05 M ammonium hydrogen carbonate with a flow of 90 ml per hour. Fractions containing the main protein peak were pooled and lyophilized. The yield was 520 mg of des(B23-B30)-human insulin.

A peptide with the sequence Gly-Phe-Phe-Tyr-Pro-Lys-Thr was synthesized on a PAM resin by means of protected symmetrical amino acid anhydrides by means of a peptide synthesis apparatus from Applied Biosystems. Finally, the peptide was cleaved from the resin by anhydrous hydrogen fluoride at 0°C, whereby the remaining protecting groups were simultaneously removed.

200 mg of des(B23-B30)-human insulin and 400 mg of peptide were dissolved in a mixture of 2.40 ml of dimethyl formamide and 1.20 ml of water and the pH-value of the mixture was adjusted 6.5 with triethyl amine. 10 mg of porcine trypsin in 0.20 ml of water was then added and the reaction mixture was left at 20°C for 4 hours. The reaction was then stopped by addition of 25 ml of 2-propanol and the precipitated proteins were isolated by centrifugation. The drained precipitate was redissolved in 10 ml of 1 M acetic acid and applied to a 2.6 x 20 cm column of Lichroprep® RP-18 (25-40 µm) previously equilibrated with 0.5 mM hydrochloric acid, 0.1 M sodium chloride in 30% (by volume) ethanol. The column was then eluted at 20°C at a flow of 20 ml per hour with the same buffer but with a linear increase of the ethanol content to 50% over 24 hours. The eluate was monitored for UV-absorption and fractions containing the main protein peak were pooled. The protein was precipitated by dilution with the same volume of water and adjustment of the pH-value to 5.5 with sodium hydroxide solution, and after standing at 4°C for 1 hour the precipitate was isolated by centrifugation and lyophilization.

The yield was 80 mg of des[Thr^{B27}]-human insulin, which was identified by sequential Edman degradation of the separated vinyl-pyridylated A- and B-chains.

EXAMPLE XIV

Formulation of injectable solution.

60 µmoles of a human insulin analogue according to the invention were dissolved in 4 ml of 0.1 M HCl and 20 ml of 1.5% m-cresol were added. The solution is now mixed with 40 ml of 4% glycerol and 20 ml of 65 mM disodium hydrogen phosphate, and the pH value was adjusted to 7.3. Finally the solution was adjusted to 100 ml with water and sterilized by filtration.

EXAMPLE XV

Evaluation of the degree of association.

A 2.6 cm x 88 cm column of Sephadex®G-75 was equilibrated with 13 mM sodium phosphate buffer pH 7.3 with a flow of 22 ml/hour. By application of des-(octapeptide-B^{23-30})-human insulin, cytochrome C, ribonuclease and mono- and dimeric myoglobin as molecular weight markers a curve representing the molecular weight as a function of the elution volume was drawn.

By application of 1 ml of solution containing 0.6 mM Zn-free human insulin or 0.6 mM insulin analogue and prepared as described in example XII it was found that Zn-free human insulin elutes as a tailing peak with an apparent molecular

weight of ≈14 kD and that the analogues prepared as described in the examples VI to X all were eluted as a symmetric peak with an apparent molecular weight of ≈5 kD.

These results indicate that human insulin analogues according to the invention are essential monomeric in solution at pH 7.3, wh reas the normal human insulin under the same conditions to a high degree appears as a mixture of dimers and higher oligomers.

EXAMPLE XVI

Evaluation of biological activity.

The biological activity in vitro was determined by measuring the binding affinity to the insulin receptors of isolated rat adipocytes and hepatocytes essentially as described in J. Gliemann, S. Gammeltoft: Diabetologia 10 (1974), 105-113.

The insulin analogues were compared to semisynthetic human insulin, the potency of which was set to 100%, and the results are shown in the table below:

	Adipocytes	Hepatocytes
des[Phe^{B25}],des[Thr^{B30}]-human insulin	223%	201%
des[Phe^{B25}]-human insulin	225%	249%
[Asp^{A21}]-des[Phe^{B25}],des[Thr^{B30}]-human insulin	250%	242%

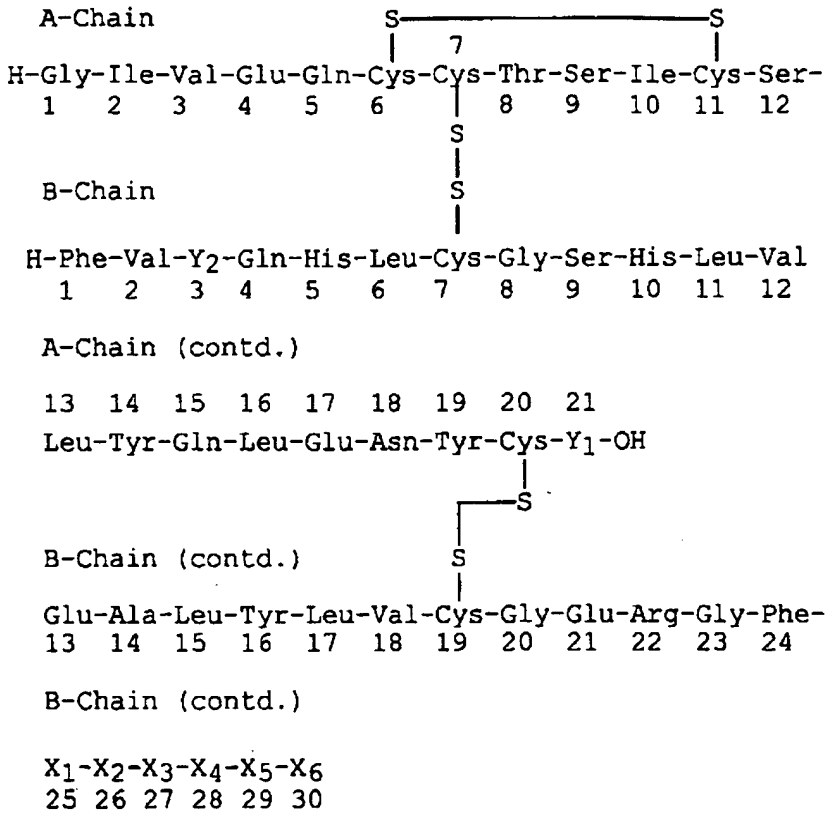
The features disclosed in the foregoing description, in the following claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

Claims

Claims for the following Contracting States : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Human insulin analogues, **characterized in** that they have a positively charged amino acid residue, i.e. Lys or Arg, in position B28, i.e. in position 8 in the B-chain calculated from [Gly^{B20}], that they optionally are further modified in the C-terminal end of the B-chain from [Phe^{B24}] to the C-terminal amino acid residue, with the proviso that there is no Pro in Position B29, and that optionally A21 and/or B3 are different from Asn.

2. Human insulin analogues, **characterized in** that they have the following formula:



wherein X_1, X_2, X_3, Y_1 and Y_2 are any naturally occurring amino acid residue; X_4 is Lys or Arg; X_5 is selected from the group consisting of any naturally occurring amino acid residue except Pro; and X_6 is any naturally occurring amino acid residue carrying the C-terminal hydroxy group or -OH or X_5 and X_6 together form the C-terminal hydroxy group.

3. Human insulin analogues according to claim 2, wherein Y_1 and/or Y_2 is selected from the group consisting of any naturally occurring amino acid residue except Asn.

4. Human insulin analogues according to claim 2, wherein

- X_1 is selected from the group consisting of Phe, Ala, His, Thr, Ser, Asn or Tyr;
- X_2 is selected from the group consisting of Tyr, Thr, Glu, Asp, Ala, His, Ser or Phe;
- X_3 is selected from the group consisting of Pro, Glu, Asp, Ser, Thr or His;
- X_5 is selected from the group consisting of Lys, Thr, Ser, Ala, Asp, or Glu;
- X_6 is selected from the group consisting of Thr-OH, Ser-OH, Ala-OH, Asp-OH, Glu-OH or -OH, or X_5 and X_6 together form the C-terminal hydroxy group;
- Y_1 is selected from the group consisting of Asn, Asp, Gly, Ser, Glu or Ala and
- Y_2 is selected from the group consisting of Asn, Gln, Glu or Asp.

5. Human insulin analogues according to claim 2, wherein

- X_1 is selected from the group consisting of Phe, Ala, His, Thr, Ser, Asn or Tyr;
- X_2 is selected from the group consisting of Tyr, Thr, Glu, Asp, Ala, His, Ser or Phe;
- X_3 is selected from the group consisting of Pro, Glu, Asp, Ser, Thr or His;
- X_5 is selected from the group consisting of Lys, Thr, Ser, Ala, Asp, or Glu;
- X_6 is -OH;

Y_1 is selected from the group consisting of Asn, Asp, Gly, Ser, Glu or Ala and
Y_2 is selected from the group consisting of Asn, Gln, Glu or Asp.

6. Human insulin analogues according to claim 2, wherein

X_1	is selected from the group consisting of Phe, Ala, His, Thr, Ser, Asn or Tyr;
X_2	is selected from the group consisting of Tyr, Thr, Glu, Asp, Ala, His, Ser or Phe;
X_3	is selected from the group consisting of Pro, Glu, Asp, Ser, Thr, or His;
X_5 and X_6	together form the C-terminal hydroxy group;
Y_1	is selected from the group consisting of Asn, Asp, Gly, Glu, Ser or Ala, and
Y_2	is selected from the group consisting of Asn, Gln, Glu or Asp.

7. Human insulin analogues according to claim 2, wherein X_1 is Phe; X_2 is Tyr; X_3 is Thr; X_5 is Lys; x_6 is Thr-OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

8. Human insulin analogues according to claim 2, wherein X_1 is Tyr; X_2 is Thr; X_3 is Pro; X_5 is Thr; X_6 is -OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

9. Human insulin analogues according to claim 2, wherein X_1 is Phe; X_2 is Thr; X_3 is Pro; X_5 is Thr; X_6 is -OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

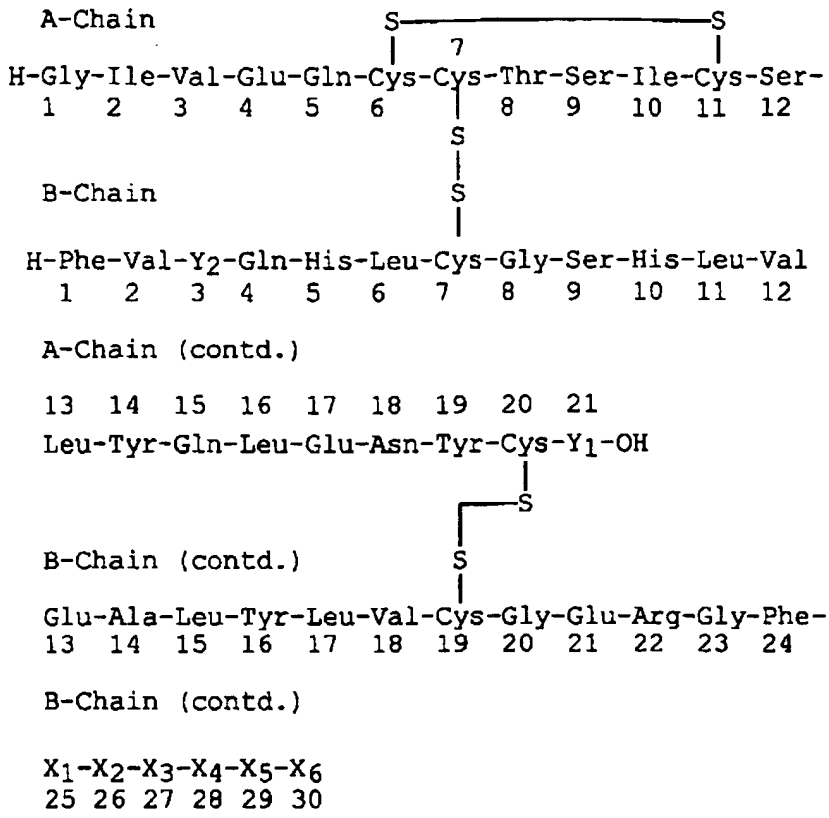
10. Human insulin analogues according to claim 2, wherein X_1 is Phe; X_2 is Tyr; X_3 is Pro; X_5 is Thr; X_6 is -OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

11. Human insulin analogues according to claim 2, wherein said X_1 amino acid is uncharged and has a carbon atom in the gamma-position which is sp2-hybridized and X_6 is -OH.

12. Human insulin analogues according to claim 11, wherein Y_1 and Y_2 are selected from the group consisting of any naturally occurring amino acid residue except Asn and X_5 is selected from the group consisting of any naturally occurring amino acid residue except Thr and Pro.

13. Human insulin analogues according to claim 11, wherein X_5 and X_6 together form -OH.

14. Pharmaceutical composition comprising a human insulin analogue having the following formula:



wherein X_1, X_2, X_3, Y_1 and Y_2 are any naturally occurring amino acid residue; X_4 is Lys or Arg; X_5 is selected from the group consisting of any naturally occurring amino acid residue except Pro; and X_6 is any naturally occurring amino acid residue carrying the C-terminal hydroxy group or -OH or X_5 and X_6 together form the C-terminal hydroxy group or a pharmaceutically acceptable salt thereof and a pharmaceutically acceptable carrier.

15. The pharmaceutical composition according to claim 14, wherein said insulin analogue exhibits low solubility at pH 7.3.

16. The pharmaceutical composition according to claim 15, wherein said insulin analogue is essentially monomeric.

17. The pharmaceutical composition according to claim 14, wherein said pharmaceutical composition is formulated as an aqueous solution.

18. The pharmaceutical composition according to claim 14, wherein said pharmaceutical composition is formulated as an aqueous suspension.

19. The pharmaceutical composition according to claim 14, wherein said pharmaceutically acceptable carrier is an aqueous, isotonic solution.

20. The pharmaceutical composition according to claim 17, 18 or 19, wherein said aqueous solution, aqueous suspension or aqueous isotonic solution further comprises zinc ions and/or a buffer, such as acetate or citrate and/or a preservative such as m-cresol, methylparaben or phenol.

21. The pharmaceutical composition of claim 14, wherein said pharmaceutical composition comprises more than one insulin analogue.

22. The pharmaceutical composition according to claim 14, wherein said pharmaceutical composition is formulated for mucosal or transcutaneous administration.

23. The pharmaceutical composition according to claim 14, wherein said pharmaceutical composition is formulated for parenteral administration.

24. The pharmaceutical composition according to claim 14, wherein X_5 is selected from the group consisting of any naturally occurring amino acid residue except Pro.

25. The pharmaceutical composition according to claim 14 or 24, wherein Y_1 and/or Y_2 is selected from the group consisting of any naturally occurring amino acid residue except Asn.

26. The pharmaceutical composition according to claim 14, wherein

X_1 is selected from the group consisting of Phe, Ala, His, Thr, Ser, Asn or Tyr;
X_2 is selected from the group consisting of Tyr, Thr, Glu, Asp, Ala, His, Ser or Phe;
X_3 is selected from the group consisting of Pro, Glu, Asp, Ser, Thr or His;
X_5 is selected from the group consisting of Lys, Thr, Ser, Ala, Asp, or Glu;
X_6 is selected from the group consisting of Thr-OH, Ser-OH, Ala-OH, Asp-OH, Glu-OH or -OH, or X_5 and X_6 together form the C-terminal hydroxy group:
Y_1 is selected from the group consisting of Asn, Asp, Gly, Ser, Glu or Ala and
Y_2 is selected from the group consisting of Asn, Gln, Glu or Asp.

27. The pharmaceutical composition according to claim 14, wherein

X_1 is selected from the group consisting of Phe, Ala, His, Thr, Ser, Asn, or Tyr;
X_2 is selected from the group consisting of Tyr, Thr, Glu, Asp, Ala, His, Ser or Phe;
X_3 is selected from the group consisting of Pro, Glu, Asp, Ser, Thr or His;
X_5 is selected from the group consisting of Lys, Thr, Ser, Ala, Asp, or Glu;
X_6 is -OH;
Y_1 is selected from the group consisting of Asn, Asp, Gly, Ser, Glu or Ala and
Y_2 is selected from the group consisting of Asn, Gln, Glu or Asp.

28. The pharmaceutical composition according to claim 14, wherein

X_1 is selected from the group consisting of Phe, Ala, His, Thr, Ser, Asn or Tyr;
X_2 is selected from the group consisting of Tyr, Thr, Glu, Asp, Ala, His, Ser or Phe;
X_3 is selected from the group consisting of Pro, Glu, Asp, Ser, Thr, or His;
X_5 and X_6 together form the C-terminal hydroxy group;
Y_1 is selected from the group consisting of Asn, Asp, Gly, Glu, Ser or Ala, and
Y_2 is selected from the group consisting of Asn, Gln, Glu or Asp.

29. The pharmaceutical composition according to claim 14, wherein X_1 is Phe; X_2 is Tyr; X_3 is Thr; X_5 is Lys; X_6 is Thr-OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

30. The pharmaceutical composition according to claim 14, wherein X_1 is Tyr; X_2 is Thr; X_3 is Pro; X_5 is Thr; X_6 is -OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

31. The pharmaceutical composition according to claim 14, wherein X_1 is Phe; X_2 is Thr; X_3 is Pro; X_5 is Thr; X_6 is -OH; Y_1 is selected from the group consisting of Asn, Asp, Ser, or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

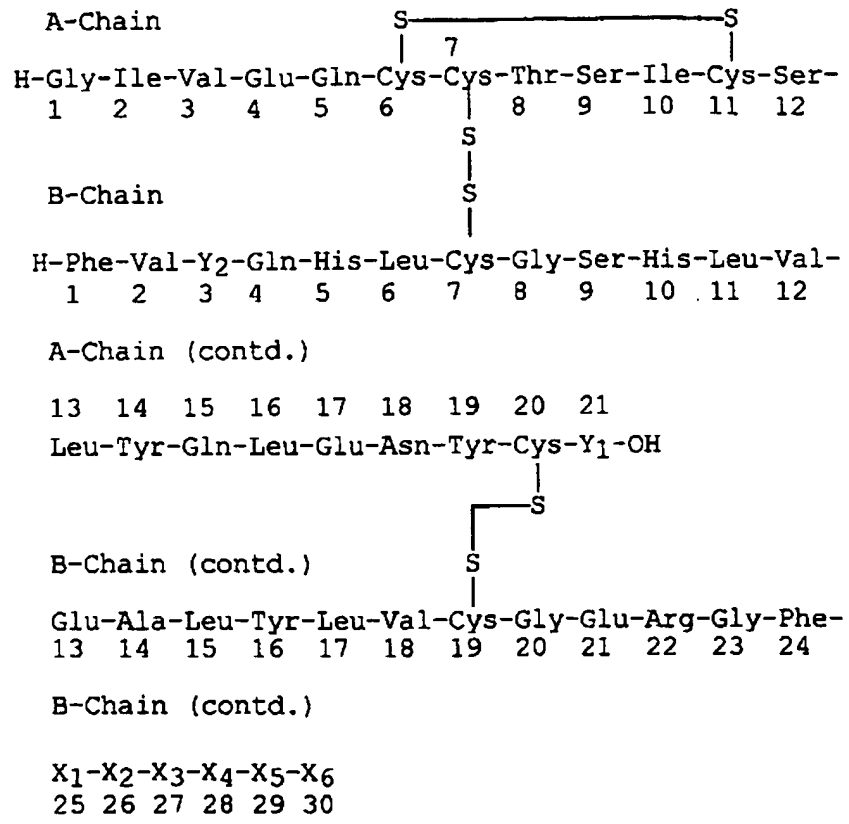
32. The pharmaceutical composition according to claim 14, wherein X_1 is Phe; X_2 is Tyr; X_3 is Pro; X_5 is Thr; X_6 is -OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

33. The pharmaceutical composition according to claim 14, wherein said X_1 amino acid is uncharged and has a carbon atom in the gamma-position which is sp2-hybridized and X_6 is -OH.

34. The pharmaceutical composition according to claim 33, wherein Y_1 and Y_2 are selected from the group consisting of any naturally occurring amino acid residue except Asn and X_5 is selected from the group consisting of any naturally occurring amino acid residue except Thr.

35. The pharmaceutical composition according to claim 33, wherein X_5 and X_6 together form -OH.

36. A process for preparing a human insulin analogue having the following formula:



wherein X_1, X_2, X_3, Y_1 and Y_2 are any naturally occurring amino acid residue; X_4 is Lys or Arg; X_5 is selected from the group consisting of any naturally occurring amino acid residue except Pro; and X_6 is any naturally occurring amino acid residue carrying the C-terminal hydroxy group or -OH or X_5 and X_6 together form the C-terminal hydroxy group, wherein a DNA sequence encoding a precursor of the insulin analogue in question is inserted into a suitable yeast expression vehicle which, when transferred to yeast, is capable of expressing and secreting the precursor of the insulin analogue in which [$LysB^{28}$], [Arg^{B28}][Lys^{B29}] or [Arg^{B29}] is connected to Gly^{A1} by a peptide bond or a peptide of the formula III

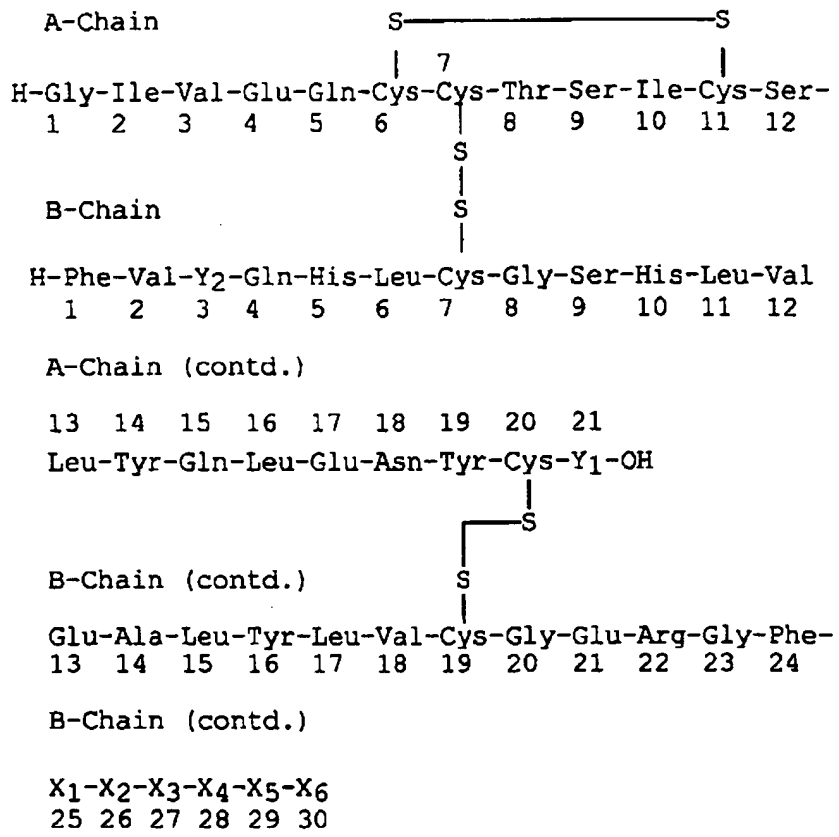
-R(n)-R(1)-

wherein R is a peptide chain with n amino acid residues, n is an integer from 0 to 33 and R(1) is Lys or Arg, the transformed yeast strain is cultured in a suitable nutrient medium, and the precursor is recovered from the culture broth and reacted with an amino compound of the formula IV

Q-QR"

wherein Q is a single amino acid residue and R" is a carboxy protecting group such as methyl or tert-butyl, using trypsin or trypsin-like enzyme as a catalyst in a mixture of water and organic solvents, whereupon the carboxy protecting group is removed and the insulin analogue is isolated from the reaction mixture, or
an insulin analogue precursor in which the C-terminal amino acid is different from Lys or Arg, said precursor having a bridge of a single pair of basic amino acids selected from the group consisting of Lys and Arg between the C-terminal and Gly^{A1} may be isolated and then converted into the insulin analogue by enzymatic treatment using trypsin and carboxypeptidase B.

37. A process for preparing a human insulin analogue having the following formula:

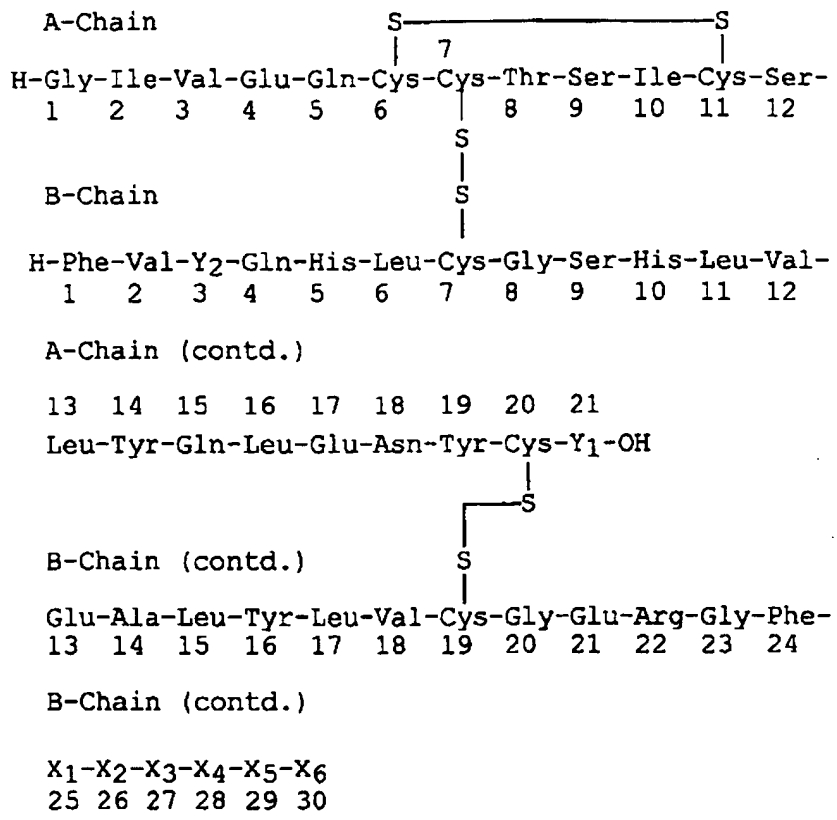


wherein X_1, X_2, X_3, Y_1 and Y_2 are any naturally occurring amino acid residue; X_4 is Lys or Arg; X_5 is selected from the group consisting of any naturally occurring amino acid residue except Pro; and X_6 is any naturally occurring amino acid residue carrying the C-terminal hydroxy group or -OH or X_5 and X_6 together form the C-terminal hydroxy group, wherein des(B23-B30)-human insulin is prepared by treating an insulin with trypsin to cleave off the (B23-B30)-amino acids, the desired peptide of six to eight amino acids is synthesized, the resulting peptide is coupled to des(B23-B30)-human insulin, and the resulting insulin analogue is isolated from the reaction mixture.

38. The use of a compound according to any of claims 1 to 13 for the preparation of a medicament for use in the treatment of diabetes.

Claims for the following Contracting State : ES

1. A process for preparing a human insulin analogue having the following formula:



wherein X_1, X_2, X_3, Y_1 and Y_2 are any naturally occurring amino acid residue; X_4 is Lys or Arg; X_5 is selected from the group consisting of any naturally occurring amino acid residue except Pro; and X_6 is any naturally occurring amino acid residue carrying the C-terminal hydroxy group or -OH or X_5 and X_6 together form the C-terminal hydroxy group, wherein a DNA sequence encoding a precursor of the insulin analogue in question is inserted into a suitable yeast expression vehicle which, when transferred to yeast, is capable of expressing and secreting the precursor of the insulin analogue in which [Lys^{B28}] , [Arg^{B28}], [Lys^{B29}] or [Arg^{B29}] is connected to Gly^{A1} by a peptide bond or a peptide of the formula III

-R(n)-R(1)-

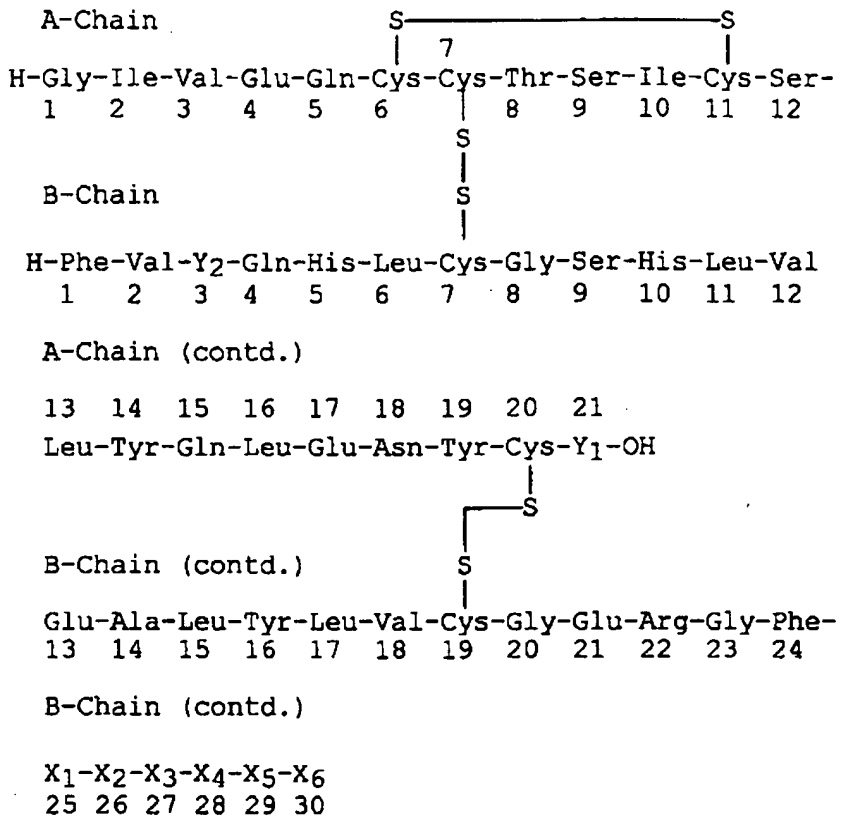
wherein R is a peptide chain with n amino acid residues, n is an integer from 0 to 33 and R(1) is Lys or Arg, the transformed yeast strain is cultured in a suitable nutrient medium, and the precursor is recovered from the culture broth and reacted with an amino compound of the formula IV

Q-QR"

wherein Q is a single amino acid residue and R" is a carboxy protecting group such as methyl or tert-butyl, using trypsin or trypsin-like enzyme as a catalyst in a mixture of water and organic solvents, whereupon the carboxy protecting group is removed and the insulin analogue is isolated from the reaction mixture, r
an insulin analogue precursor in which the C-terminal amino acid is different from Lys or Arg, said precursor having

a bridge of a single pair of basic amino acids selected from the group consisting of Lys and Arg between the C-terminal and Gly^{A1} may be isolated and then converted into the insulin analogue by enzymatic treatment using trypsin and carboxypeptidase B.

2. A process for preparing a human insulin analogue having the following formula:



wherein X_1, X_2, X_3, Y_1 and Y_2 are any naturally occurring amino acid residue; X_4 is Lys or Arg; X_5 is selected from the group consisting of any naturally occurring amino acid residue except Pro; and X_6 is any naturally occurring amino acid residue carrying the C-terminal hydroxy group or -OH or X_5 and X_6 together form the C-terminal hydroxy group, wherein des(B23-B30)-human insulin is prepared by treating an insulin with trypsin to cleave off the (B23-B30)-amino acids, the desired peptide of six to eight amino acids is synthesized, the resulting peptide is coupled to des(B23-B30)-human insulin, and the resulting insulin analogue is isolated from the reaction mixture.

3. The process according to claim 1 or 2, wherein Y_1 and/or Y_2 is selected from the group consisting of any naturally occurring amino acid residue except Asn.

4. The process according to claim 1 or 2, wherein

- X_1 is selected from the group consisting of Phe, Ala, His, Thr, Ser, Asn or Tyr;
- X_2 is selected from the group consisting of Tyr, Thr, Glu, Asp, Ala, His, Ser or Phe;
- X_3 is selected from the group consisting of Pro, Glu, Asp, Ser, Thr or His;
- X_5 is selected from the group consisting of Lys, Thr, Ser, Ala, Asp, or Glu;
- X_6 is selected from the group consisting of Thr-OH, Ser-OH, Ala-OH, Asp-OH, Glu-OH or -OH, or X_5 and X_6 together form the C-terminal hydroxy group;
- Y_1 is selected from the group consisting of Asn, Asp, Gly, Ser, Glu or Ala and
- Y_2 is selected from the group consisting of Asn, Gln, Glu or Asp.

5. The process according to claim 1 or 2, wherein

X_1 is selected from the group consisting of Phe, Ala, His, Thr, Ser, Asn or Tyr;
X_2 is selected from the group consisting of Tyr, Thr, Glu, Asp, Ala, His, Ser or Phe;
X_3 is selected from the group consisting of Pr , Glu, Asp, Ser, Thr or His;
X_5 is selected from the group consisting of Lys, Thr, Ser, Ala, Asp, or Glu;
X_6 is -OH;
Y_1 is selected from the group consisting of Asn, Asp, Gly, Ser, Glu or Ala and
Y_2 is selected from the group consisting of Asn, Gln, Glu or Asp.

6. The process according to claim 1 or 2, wherein

X_1	is selected from the group consisting of Phe, Ala, His, Thr, Ser, Asn or Tyr;
X_2	is selected from the group consisting of Tyr, Thr, Glu, Asp, Ala, His, Ser or Phe;
X_3	is selected from the group consisting of Pro, Glu, Asp, Ser, Thr, or His;
X_5 and X_6	together form the C-terminal hydroxy group;
Y_1	is selected from the group consisting of Asn, Asp, Gly, Glu, Ser or Ala, and
Y_2	is selected from the group consisting of Asn, Gln, Glu or Asp.

7. The process according to claim 1 or 2, wherein X_1 is Phe; X_2 is Tyr; X_3 is Thr; X_5 is Lys; X_6 is Thr-OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y2 is selected from the group consisting of Asn, Gln, Asp or Glu.

8. The process according to claim 1 or 2, wherein X_1 is Tyr; X_2 is Thr; X_3 is Pro; X_5 is Thr; X_6 is -OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y2 is selected from the group consisting of Asn, Gln, Asp or Glu.

9. The process according to claim 1 or 2, wherein X_1 is Phe; X_2 is Thr; X_3 is Pro; X_5 is Thr; X_6 is -OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

10. The process according to claim 1 or 2, wherein X_1 is Phe; X_2 is Tyr; X_3 is Pro; X_5 is Thr; X_6 is -OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

11. The process according to claim 1 or 2, wherein said X_1 amino acid is uncharged and has a carbon atom in the gamma-position which is sp2-hybridized and X_6 is -OH.

12. The process according to claim 11, wherein Y1 and Y2 are selected from the group consisting of any naturally occurring amino acid residue except Asn and X_5 is selected from the group consisting of any naturally occurring amino acid residue except Thr and Pro.

13. The process according to claim 11, wherein X_5 and X_6 together form -OH.

14. A process for preparing a pharmaceutical composition comprising formulating a human insulin analogue prepared by the process according to any of claims 1 to 13 or a pharmaceutically acceptable salt thereof with a pharmaceutically acceptable carrier.

15. The process according to claim 14, wherein said insulin analogue exhibits low solubility at pH 7.3.

16. The process according to claim 15, wherein said insulin analogue is essentially monomeric.

17. The process according to claim 14, wherein said pharmaceutical composition is formulated as an aqueous solution.

18. The process according to claim 14, wherein said pharmaceutical composition is formulated as an aqueous suspension.

19. The process according to claim 14, wherein said pharmaceutically acceptable carrier is an aqueous, isotonic so-

lution.

20. The process according to claim 17, 18 or 19, wherein said aqueous solution, aqueous suspension or aqueous isotonic solution further comprises zinc ions and/or a buffer, such as acetate or citrate and/or a preservative such as m-cresol, methylparaben or phenol.

21. The process of claim 14, wherein said pharmaceutical composition comprises more than one insulin analogue.

22. The process according to claim 14, wherein said pharmaceutical composition is formulated for mucosal or transcutaneous administration.

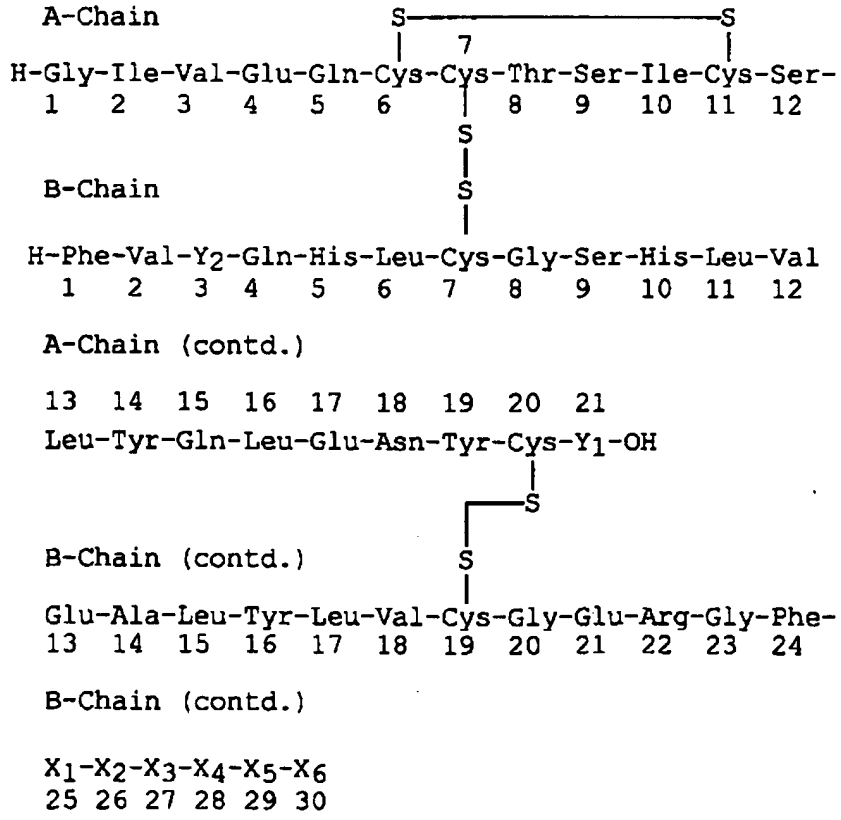
23. The process according to claim 14, wherein said pharmaceutical composition is formulated for parenteral administration.

24. The use of a compound prepared in the process according to any of claims 1 to 13 for the preparation of a medicament for use in the treatment of diabetes.

Claims for the following Contracting State : GR

1. Human insulin analogues, **characterized in** that they have a positively charged amino acid residue, i.e. Lys or Arg, in position B28, i.e. in position 8 in the B-chain calculated from [Gly^{B20}], that they optionally are further modified in the C-terminal end of the B-chain from [Phe^{B24}] to the C-terminal amino acid residue, with the proviso that there is no Pro in Position B29, and that optionally A21 and/or B3 are different from Asn.

2. Human insulin analogues, **characterized in** that they have the following formula:



wherein X_1, X_2, X_3, Y_1 and Y_2 are any naturally occurring amino acid residue; X_4 is Lys or Arg; X_5 is selected from the group consisting of any naturally occurring amino acid residue except Pro; and X_6 is any naturally occurring amino acid residue carrying the C-terminal hydroxy group or -OH or X_5 and X_6 together form the C-terminal hydroxy group.

3. Human insulin analogues according to claim 2, wherein Y_1 and/or Y_2 is selected from the group consisting of any naturally occurring amino acid residue except Asn.

4. Human insulin analogues according to claim 2, wherein

X_1 is selected from the group consisting of Phe, Ala, His, Thr, Ser, Asn or Tyr;
X_2 is selected from the group consisting of Tyr, Thr, Glu, Asp, Ala, His, Ser or Phe;
X_3 is selected from the group consisting of Pro, Glu, Asp, Ser, Thr or His;
X_5 is selected from the group consisting of Lys, Thr, Ser, Ala, Asp, or Glu;
X_6 is selected from the group consisting of Thr-OH, Ser-OH, Ala-OH, Asp-OH, Glu-OH or -OH, or X_5 and X_6 together form the C-terminal hydroxy group;
Y_1 is selected from the group consisting of Asn, Asp, Gly, Ser, Glu or Ala and
Y_2 is selected from the group consisting of Asn, Gln, Glu or Asp.

5. Human insulin analogues according to claim 2, wherein

X_1 is selected from the group consisting of Phe, Ala, His, Thr, Ser, Asn or Tyr;
X_2 is selected from the group consisting of Tyr, Thr, Glu, Asp, Ala, His, Ser or Phe;
X_3 is selected from the group consisting of Pro, Glu, Asp, Ser, Thr or His;
X_5 is selected from the group consisting of Lys, Thr, Ser, Ala, Asp, or Glu;
X_6 is -OH;
Y_1 is selected from the group consisting of Asn, Asp, Gly, Ser, Glu or Ala and
Y_2 is selected from the group consisting of Asn, Gln, Glu or Asp.

6. Human insulin analogues according to claim 2, wherein

X_1	is selected from the group consisting of Phe, Ala, His, Thr, Ser, Asn or Tyr;
X_2	is selected from the group consisting of Tyr, Thr, Glu, Asp, Ala, His, Ser or Phe;
X_3	is selected from the group consisting of Pro, Glu, Asp, Ser, Thr, or His;
X_5 and X_6	together form the C-terminal hydroxy group;
Y_1	is selected from the group consisting of Asn, Asp, Gly, Glu, Ser or Ala, and
Y_2	is selected from the group consisting of Asn, Gln, Glu or Asp.

7. Human insulin analogues according to claim 2, wherein X_1 is Phe; X_2 is Tyr; X_3 is Thr; X_5 is Lys; X_6 is Thr-OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

8. Human insulin analogues according to claim 2, wherein X_1 is Tyr; X_2 is Thr; X_3 is Pro; X_5 is Thr; X_6 is -OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

9. Human insulin analogues according to claim 2, wherein X_1 is Phe; X_2 is Thr; X_3 is Pro; X_5 is Thr; X_6 is -OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

10. Human insulin analogues according to claim 2, wherein X_1 is Phe; X_2 is Tyr; X_3 is Pro; X_5 is Thr; X_6 is -OH; Y_1 is selected from the group consisting of Asn, Asp, Ser or Gly and Y_2 is selected from the group consisting of Asn, Gln, Asp or Glu.

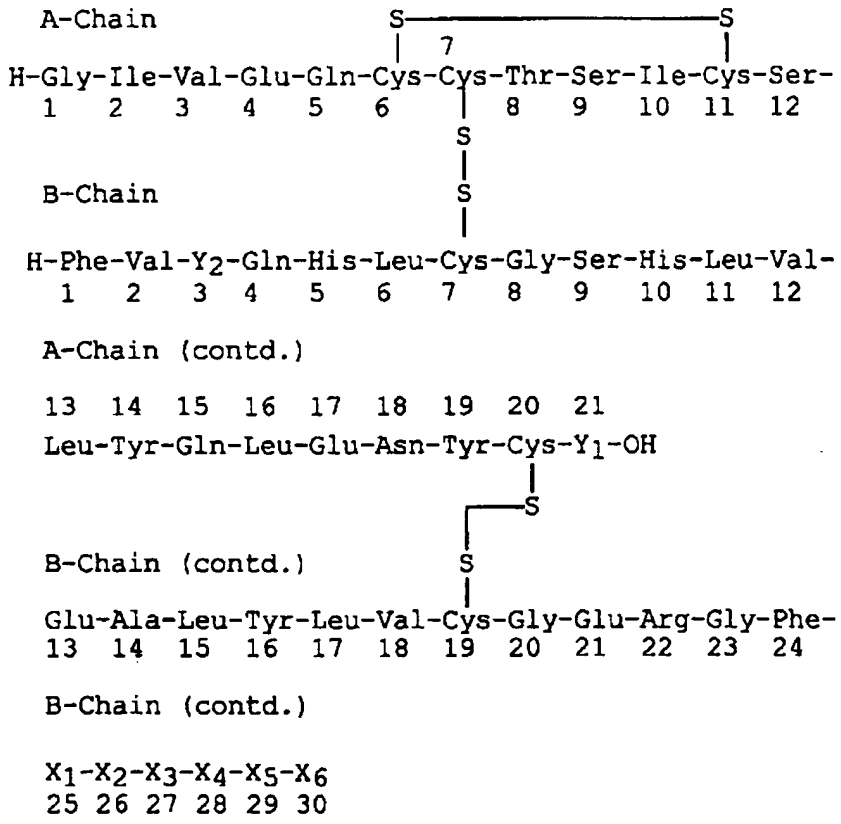
11. Human insulin analogues according to claim 2, wherein said X_1 amino acid is uncharged and has a carbon atom in the gamma-position which is sp2-hybridized and X_6 is -OH.

12. Human insulin analogues according to claim 11, wherein Y_1 and Y_2 are selected from the group consisting of any

naturally occurring amino acid residue except Asn and X_5 is selected from the group consisting of any naturally occurring amino acid residue except Thr and Pro.

13. Human insulin analogues according to claim 11, wherein X_5 and X_6 together form -OH.

14. A process for preparing a human insulin analogue having the following formula:



wherein X_1, X_2, X_3, Y_1 and Y_2 are any naturally occurring amino acid residue; X_4 is Lys or Arg; X_5 is selected from the group consisting of any naturally occurring amino acid residue except Pro; and X_6 is any naturally occurring amino acid residue carrying the C-terminal hydroxy group or -OH or X_5 and X_6 together form the C-terminal hydroxy group, wherein a DNA sequence encoding a precursor of the insulin analogue in question is inserted into a suitable yeast expression vehicle which, when transferred to yeast, is capable of expressing and secreting the precursor of the insulin analogue in which [$LysB^{28}$], [Arg^{B28}], [Lys^{B29}] or [Arg^{B29}] is connected to Gly^{A1} by a peptide bond or a peptide of the formula III

-R(n)-R(1)-

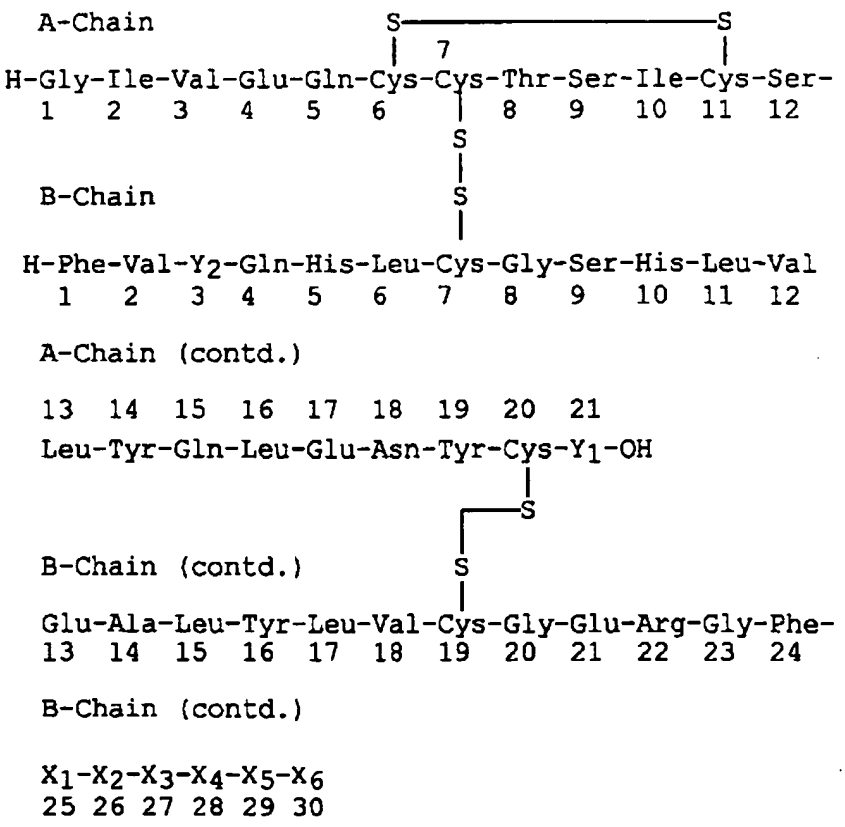
wherein R is a peptide chain with n amino acid residues, n is an integer from 0 to 33 and R(1) is Lys or Arg, the transformed yeast strain is cultured in a suitable nutrient medium, and the precursor is recovered from the culture broth and reacted with an amino compound of the formula IV

Q-QR'

wherein Q is a single amino acid residue and R' is a carboxy protecting group such as methyl or tert-butyl, using

trypsin or trypsin-like enzyme as a catalyst in a mixture of water and organic solvents, whereupon the carboxy protecting group is removed and the insulin analogue is isolated from the reaction mixture, or
an insulin analogue precursor in which the C-terminal amino acid is different from Lys or Arg, said precursor having a bridge of a single pair of basic amino acids selected from the group consisting of Lys and Arg between the C-terminal and Gly^{A1} may be isolated and then converted into the insulin analogue by enzymatic treatment using trypsin and carboxypeptidase B.

15. A process for preparing a human insulin analogue having the following formula:



wherein X_1, X_2, X_3, Y_1 and Y_2 are any naturally occurring amino acid residue; X_4 is Lys or Arg; X_5 is selected from the group consisting of any naturally occurring amino acid residue except Pro; and X_6 is any naturally occurring amino acid residue carrying the C-terminal hydroxy group or -OH or X_5 and X_6 together form the C-terminal hydroxy group, wherein des(B23-B30)-human insulin is prepared by treating an insulin with trypsin to cleave off the (B23-B30)-amino acids, the desired peptide of six to eight amino acids is synthesized, the resulting peptide is coupled to des(B23-B30)-human insulin, and the resulting insulin analogue is isolated from the reaction mixture.

16. A process for preparing a pharmaceutical composition comprising formulating a human insulin analogue according to any of claims 1 to 13 or a pharmaceutically acceptable salt thereof with a pharmaceutically acceptable carrier.

17. The process according to claim 16, wherein said insulin analogue exhibits low solubility at pH 7.3.

18. The process according to claim 17, wherein said insulin analogue is essentially monomeric.

19. The process according to claim 16, wherein said pharmaceutical composition is formulated as an aqueous solution.

20. The process according to claim 16, wherein said pharmaceutical composition is formulated as an aqueous suspension.

21. The process according to claim 16, wherein said pharmaceutically acceptable carrier is an aqueous, isotonic solution.

22. The process according to claim 19, 20 or 21, wherein said aqueous solution, aqueous suspension or aqueous isotonic solution further comprises zinc ions and/or a buffer, such as acetate or citrate and/or a preservative such as m-cresol, methylparaben or phenol.

23. The process of claim 16, wherein said pharmaceutical composition comprises more than one insulin analogue.

24. The process according to claim 16, wherein said pharmaceutical composition is formulated for mucosal or transcutaneous administration.

25. The process according to claim 16, wherein said pharmaceutical composition is formulated for parenteral administration.

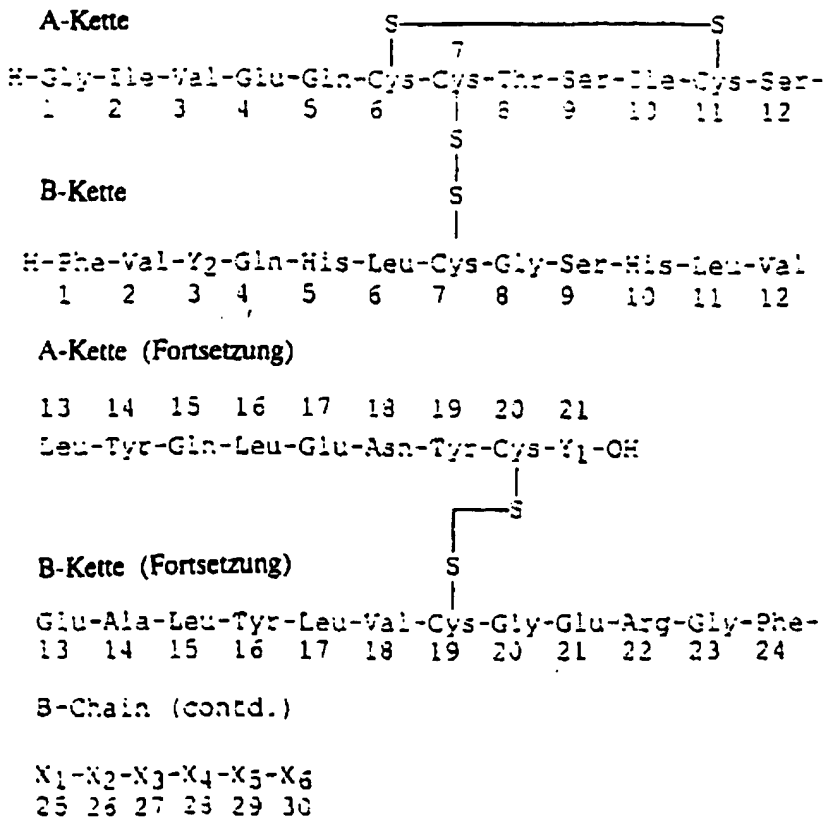
26. The use of a compound according to any of claims 1 to 13 for the preparation of a medicament for use in the treatment of diabetes.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Analoge von menschlichem Insulin, gekennzeichnet dadurch, daß sie einen positiv geladenen Aminosäurerest, d.h. Lys oder Arg, an Position B28 besitzen, d.h. in Position 8 in der B-Kette, gerechnet von [Gly^{B20}], daß sie wahlweise zusätzlich in dem C-terminalen Ende der B-Kette von [Phe^{B24}] bis zu dem C-terminalen Aminosäurerest modifiziert sind, mit der Maßgabe, daß sich kein Pro in Position B29 befindet, und daß wahlweise A21 und/oder B3 nicht Asn sind.

2. Analoge von menschlichem Insulin, dadurch gekennzeichnet, daß sie die folgende Formel besitzen:



wobei X_1, X_2, X_3, Y_1 und Y_2 jede beliebige, natürlich vorkommende Aminosäure sind; X_4, Lys oder Arg ist; X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen, natürlich vorkommenden Aminosäure mit Ausnahme von Pro; und X_6 jede beliebige natürlich vorkommende Aminosäure ist, die die C-terminale Hydroxygruppe trägt oder -OH oder X_5 und X_6 zusammen die C-terminale Hydroxygruppe bilden.

3. Analoge von menschlichem Insulin nach Anspruch 2, wobei Y_1 und/-oder Y_2 ausgewählt sind aus der Gruppe, bestehend aus jeder beliebigen, natürlich vorkommenden Aminosäure mit Ausnahme von Asn.

4. Analoge von menschlichem Insulin nach Anspruch 2, wobei

   X_1 ausgewählt ist aus der Gruppe, bestehend aus Phe, Ala, His, Thr, Ser, Asn oder Tyr;
   X_2 ausgewählt ist aus der Gruppe, bestehend aus Tyr, Thr, Glu, Asp, Ala, His, Ser oder Phe;
   X_3 ausgewählt ist aus der Gruppe, bestehend aus Pro, Glu, Asp, Ser, Thr oder His;
   X_5 ausgewählt ist aus der Gruppe, bestehend aus Lys, Thr, Ser, Ala, Asp oder Glu;
   X_6 ausgewählt ist aus der Gruppe, bestehend aus Thr-OH, Ser-OH, Ala-OH, Asp-OH, Glu-OH oder -OH, oder X_5 und X_6 bilden zusammen die C-terminale Hydroxygruppe;
   Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Gly, Ser, Glu oder Ala und
   Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Glu oder Asp.

5. Analoge von menschlichem Insulin nach Anspruch 2, wobei

   X_1 ausgewählt ist aus der Gruppe, bestehend aus Phe, Ala, His, Thr, Ser, Asn oder Tyr;
   X_2 ausgewählt ist aus der Gruppe, bestehend aus Tyr, Thr, Glu, Asp, Ala, His, Ser oder Phe;
   X_3 ausgewählt ist aus der Gruppe, bestehend aus Pro, Glu, Asp, Ser, Thr oder His;
   X_5 ausgewählt ist aus der Gruppe, bestehend aus Lys, Thr, Ser, Ala, Asp oder Glu;
   X_6 -OH ist;
   Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Gly, Ser, Glu oder Ala und

Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Glu oder Asp.

6. Analoge von menschlichem Insulin nach Anspruch 2, wobei

X_1	ausgewählt ist aus der Gruppe, bestehend aus Phe, Ala, His, Thr, Ser, Asn oder Tyr;
X_2	ausgewählt ist aus der Gruppe, bestehend aus Tyr, Thr, Glu, Asp, Ala, His, Ser oder Phe;
X_3	ausgewählt ist aus der Gruppe, bestehend aus Pro, Glu, Asp, Ser, Thr oder His;
X_5 und X_6	zusammen die C-terminale Hydroxygruppe bilden;
Y_1	ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Gly, Glu, Ser oder Ala, und
Y_2	ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Glu oder Asp.

7. Analoge von menschlichem Insulin nach Anspruch 2, wobei
X_1 Phe ist; X_2 Tyr ist; X_3 Thr ist; X_5 Lys ist; X_6 Thr-OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

8. Analoge von menschlichem Insulin nach Anspruch 2, wobei X_1 Tyr ist; X_2 Thr ist; X_3 Pro ist; X_5 Thr ist; X_6 -OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

9. Analoge von menschlichem Insulin nach Anspruch 2, wobei X_1 Phe ist; X_2 Thr ist; X_3 Pro ist; X_5 Thr ist; X_6 -OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

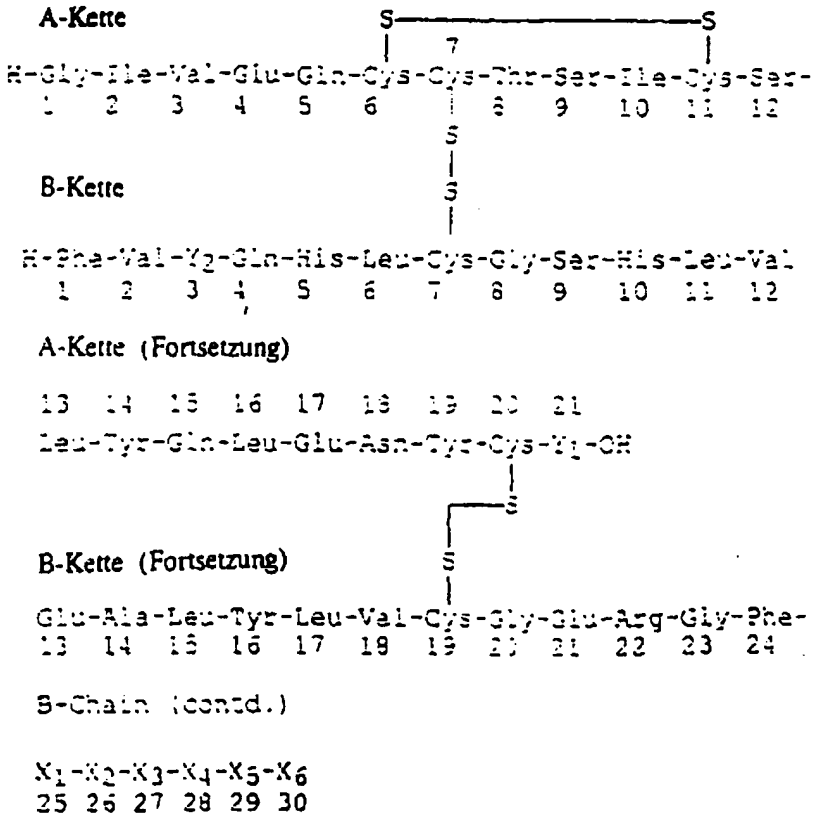
10. Analoge von menschlichem Insulin nach Anspruch 2, wobei X_1 Phe ist; X_2 Tyr ist; X_3 Pro ist; X_5 Thr ist; X_6 -OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

11. Analoge von menschlichem Insulin nach Anspruch 2, wobei die Aminosäure X_1 keine Ladung besitzt und ein Kohlenstoffatom in der Gamma-Position besitzt, das sp^2-hybridisiert ist und X_6 -OH ist.

12. Analoge von menschlichem Insulin nach Anspruch 11, wobei Y_1 und Y_2 ausgewählt sind aus der Gruppe, bestehend aus jeder beliebigen natürlich vorkommenden Aminosäure mit Ausnahme von Asn und X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen natürlich vorkommenden Aminosäure mit Ausnahme von Thr und Pro.

13. Analoge von menschlichem Insulin nach Anspruch 11, wobei X_5 und X_6 zusammen -OH bilden.

14. Pharmazeutische Zusammensetzung, enthaltend ein Analoges von menschlichem Insulin mit der folgenden Formel:



wobei X_1, X_2, X_3, Y_1 und Y_2 jede beliebige natürlich vorkommende Aminosäure sind; X_4 Lys oder Arg ist; X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen natürlich vorkommenden Aminosäure mit Ausnahme von Pro; und X_6 jede beliebige, natürlich vorkommende Aminosäure ist, die die C-terminale Hydroxygruppe trägt, oder -OH ist oder X_5 und X_6 zusammen die C-terminale Hydroxygruppe bilden, oder ein pharmazeutisch verträgliches Salz davon und ein pharmazeutisch verträglicher Träger ist.

15. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei das Insulin-Analoge eine geringe Löslichkeit bei pH 7,3 aufweist.

16. Pharmazeutische Zusammensetzung nach Anspruch 15, wobei das Insulin-Analoge im wesentlichen monomer ist.

17. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei die pharmazeutische Zusammensetzung als eine wäßrige Lösung formuliert ist.

18. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei die pharmazeutische Zusammensetzung als eine wäßrige Suspension formuliert ist.

19. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei der pharmazeutisch verträgliche Träger eine wäßrige isotonische Lösung ist.

20. Pharmazeutische Zusammensetzung nach Anspruch 17, 18 oder 19, wobei die wäßrige Lösung, wäßrige Suspension oder wäßrige isotonische Lösung weiterhin Zinkionen und/oder einen Puffer umfaßt wie Acetat oder Citrat und/oder einen Konservierungsstoff wie m-Kresol, Methylparaben oder Phenol.

21. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei die pharmazeutische Zusammensetzung mehr

als ein Insulin-Analoges umfaßt.

22. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei die pharmazeutische Zusammensetzung für eine mukosale oder transkutane Verabreichung formuliert ist.

23. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei die pharmazeutische Zusammensetzung für eine parenterale Verabreichung formuliert ist.

24. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen, natürlich vorkommenden Aminosäure mit Ausnahme von Pro.

25. Pharmazeutische Zusammensetzung nach Anspruch 14 oder 24, wobei Y_1 und/oder Y_2 ausgewählt sind aus der Gruppe, bestehend aus jeder natürlich vorkommenden Aminosäure mit Ausnahme von Asn.

26. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei

X_1 ausgewählt ist aus der Gruppe, bestehend aus Phe, Ala, His, Thr, Ser, Asn oder Tyr;
X_2 ausgewählt ist aus der Gruppe, bestehend aus Tyr, Thr, Glu, Asp, Ala, His, Ser oder Phe;
X_3 ausgewählt ist aus der Gruppe, bestehend aus Pro, Glu, Asp, Ser, Thr oder His;
X_5 ausgewählt ist aus der Gruppe, bestehend aus Lys, Thr, Ser, Ala, Asp oder Glu;
X_6 ausgewählt ist aus der Gruppe, bestehend aus Thr-OH, Ser-OH, Ala-OH, Asp-OH, Glu-OH oder -OH, oder X_5 und X_6 bilden zusammen die C-terminale Hydroxygruppe;
Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Gly, Ser, Glu oder Ala und
Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Glu oder Asp.

27. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei

X_1 ausgewählt ist aus der Gruppe, bestehend aus Phe, Ala, His, Thr, Ser, Asn oder Tyr;
X_2 ausgewählt ist aus der Gruppe, bestehend aus Tyr, Thr, Glu, Asp, Ala, His, Ser oder Phe;
X_3 ausgewählt ist aus der Gruppe, bestehend aus Pro, Glu, Asp, Ser, Thr oder His;
X_5 ausgewählt ist aus der Gruppe, bestehend aus Lys, Thr, Ser, Ala, Asp oder Glu;
X_6 -OH ist;
Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Gly, Ser, Glu oder Ala und
Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Glu oder Asp.

28. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei

X_1	ausgewählt ist aus der Gruppe, bestehend aus Phe, Ala, His, Thr, Ser, Asn oder Tyr;
X_2	ausgewählt ist aus der Gruppe, bestehend aus Tyr, Thr, Glu, Asp, Ala, His, Ser oder Phe;
X_3	ausgewählt ist aus der Gruppe, bestehend aus Pro, Glu, Asp, Ser, Thr oder His;
X_5 und X_6	zusammen die C-terminale Hydroxygruppe bilden;
Y_1	ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Gly, Glu, Ser oder Ala und
Y_2	ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Glu oder Asp.

29. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei X_1 Phe ist; X_2 Tyr ist; X_3 Thr ist; X_5 Lys ist; X_6 Thr-OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

30. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei X_1 Tyr ist; X_2 Thr ist; X_3 Pro ist; X_5 Thr ist; X_6 -OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

31. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei X_1 Phe ist; X_2 Thr ist; X_3 Pro ist; X_5 Thr ist; X_6 -OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

32. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei X_1 Phe ist; X_2 Tyr ist; X_3 Pro ist; X_5 Thr ist; X_6 -OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der

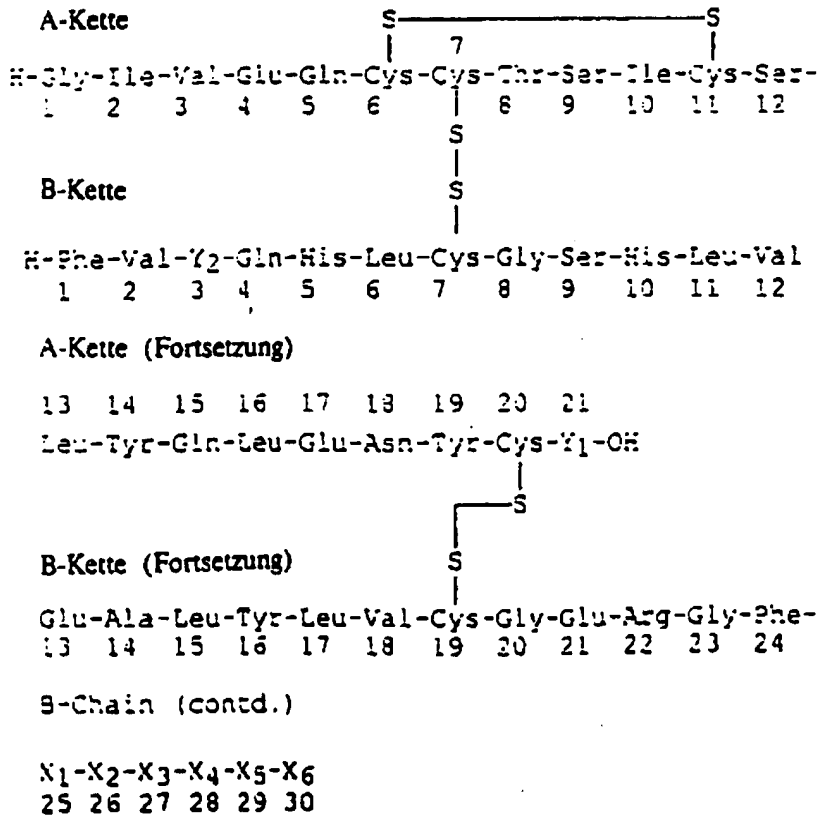
Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

33. Pharmazeutische Zusammensetzung nach Anspruch 14, wobei die Aminosäure X_1 keine Ladung besitzt und ein Kohlenstoffatom in der Gamma-Position besitzt, das sp^2-hybridisiert ist und X_6 -OH ist.

34. Pharmazeutische Zusammensetzung nach Anspruch 33, wobei Y_1 und Y_2 ausgewählt sind aus der Gruppe, bestehend aus jeder beliebigen natürlich vorkommenden Aminosäure mit Ausnahme von Asn und X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen natürlich vorkommenden Aminosäure mit Ausnahme von Thr.

35. Pharmazeutische Zusammensetzung nach Anspruch 33, wobei X_5 und X_6 zusammen -OH bilden.

36. Verfahren zur Herstellung eines Analogen von menschlichem Insulin mit der folgenden Formel:



wobei X_1, X_2, X_3, Y_1 und Y_2 jede beliebige, natürlich vorkommende Aminosäure sind; X_4 Lys oder Arg ist; X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen, natürlich vorkommenden Aminosäure mit Ausnahme von Pro; und X_6 jede beliebige natürlich vorkommende Aminosäure ist, die die C-terminale Hydroxygruppe trägt, oder -OH oder X_5 und X_6 zusammen die C-terminale Hydroxygruppe bilden, wobei eine DNA-Sequenz, die ein Vorläufermolekül des Insulin--Analogen kodiert, in ein geeignetes Hefe-Expressions-Vehikel insertiert ist, das, wenn es in Hefe überführt wird, in der Lage ist, den Vorläufer des Insulin-Analogen zu exprimieren und zu sekretieren, in dem [Lys^{B28}], [Arg^{B29}], [Lys^{B28}] oder [Arg^{B29}] mit Gly^{A1} verbunden ist durch eine Peptidbindung oder ein Peptid der Formel III

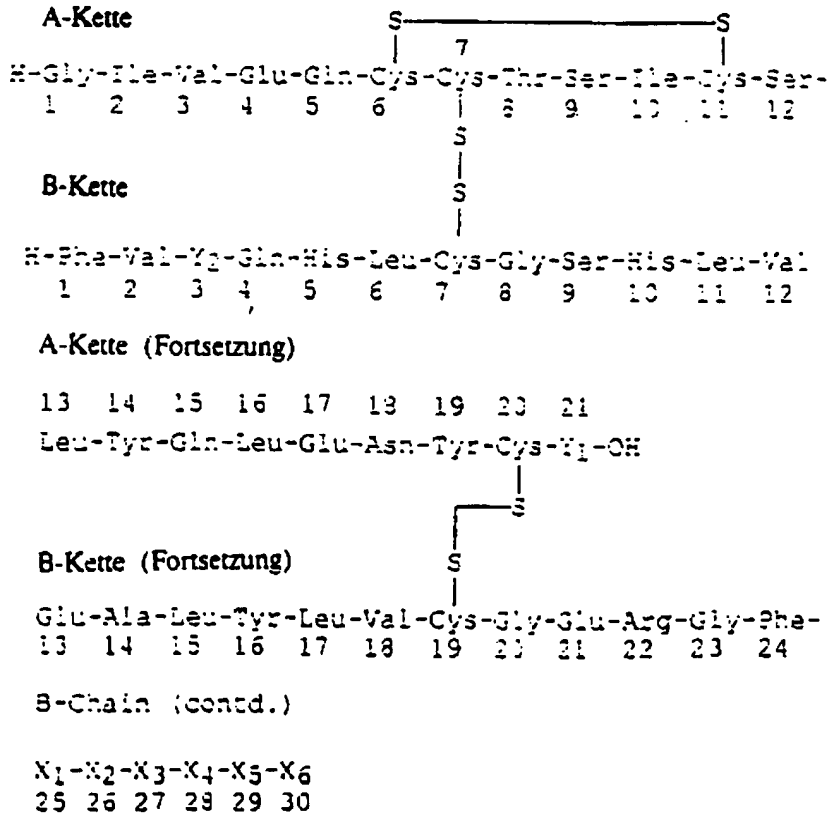
-R(n)-R(1)-

wobei R eine Peptidkette ist mit n Aminosäureresten, n eine ganze Zahl von 0 bis 33 und R(1) Lys oder Arg ist, der transformierte Hefestamm in einem geeigneten Nährmedium gezüchtet wird, und der Vorläufer aus dem Kulturmedium wiedergewonnen und mit einer Aminoverbindung der Formel IV

Q-QR"

umgesetzt wird, wobei Q ein einzelner Aminosäurerest ist und R" eine Carboxy-Schutzgruppe wie Methyl oder tert-Butyl ist, unter Verwendung von Trypsin oder einem Trypsin-artigen Enzym als Katalysator in einem Gemisch von Wasser und organischen Lösungsmitteln, woraufhin die Carboxy-Schutzgruppe entfernt wird und das Insulin-Analoge aus dem Reaktionsgemisch isoliert wird, oder ein Vorläufer eines Insulin-Analogen, in dem die C-terminale Aminosäure nicht Lys oder Arg ist, wobei der Vorläufer eine Brücke aus einem einzelnen Paar von basischen Aminosäuren. ausgewählt aus der Gruppe, bestehend aus Lys und Arg, zwischen dem C-terminalen und Gly^{A1} besitzt, isoliert werden kann und dann durch enzymatische Behandlung unter Verwendung von Trypsin oder Carboxypeptidase B in das Insulin-Analoge umgewandelt werden kann.

37. Verfahren zur Herstellung eines Analogen von menschlichem Insulin mit der folgenden Formel:

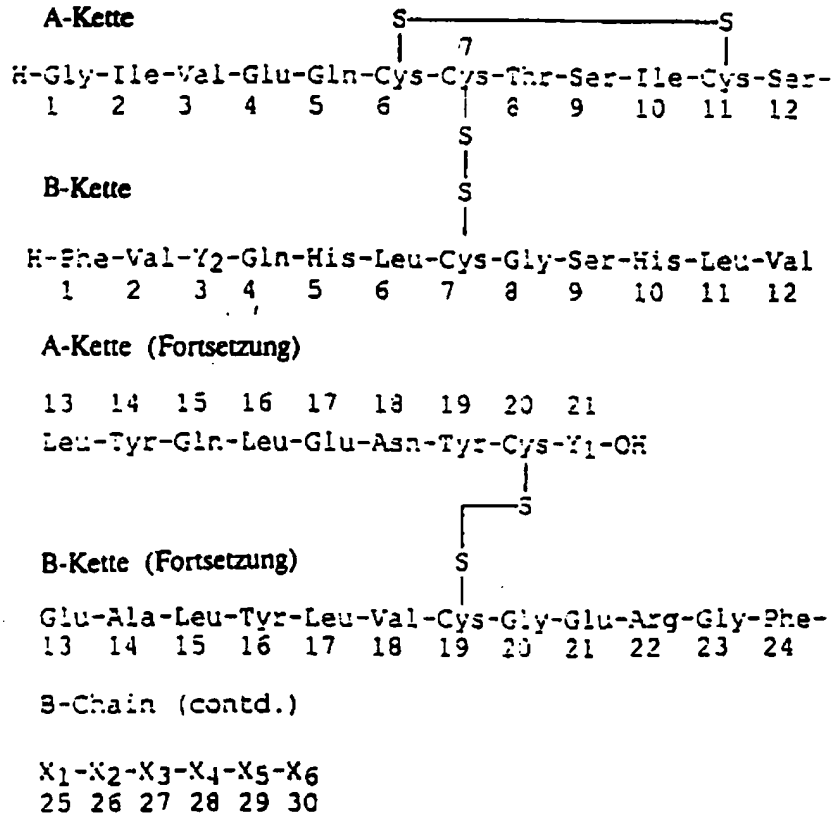


wobei X_1, X_2, X_3, Y_1 und Y_2 jede beliebige, natürlich vorkommende Aminosäure sind; X_4 Lys oder Arg ist; X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen, natürlich vorkommenden Aminosäure mit Ausnahme von Pro; und X_6 jede beliebige natürlich vorkommende Aminosäure ist, die die C-terminale Hydroxygruppe trägt, oder -OH oder X_5 und X_6 zusammen die C-terminale Hydroxygruppe bilden, wobei menschliches Des (B23-B30)-Insulin hergestellt wird durch Behandeln eines Insulins mit Trypsin unter Abspaltung der (B23-B30)-Aminosäuren, das gewünschte Peptid von sechs bis acht Aminosäuren synthetisiert wird, das resultierende Peptid an menschliches Des(B23-B30)-Insulin gekoppelt wird und das resultierende Insulin-Analoge aus dem Reaktionsgemisch isoliert wird.

38. Verwendung einer Verbindung nach einem der Ansprüche 1 bis 13 zur Herstellung eines Medikaments zur Verwendung bei der Behandlung von Diabetes.

Patentansprüche für folgenden Vertragsstaat : ES

1. Verfahren zur Herstellung eines Analogen von menschlichem Insulin mit der folgenden Formel:



wobei X_1, X_2, X_3, Y_1 und Y_2 jede beliebige, natürlich vorkommende Aminosäure sind; X_4 Lys oder Arg ist; X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen, natürlich vorkommenden Aminosäure mit Ausnahme von Pro; und X_6 jede beliebige natürlich vorkommende Aminosäure ist, die die C-terminale Hydroxygruppe trägt, oder -OH oder X_5 und X_6 zusammen die C-terminale Hydroxygruppe bilden, wobei eine DNA-Sequenz, die ein Vorläufermolekül des Insulin--Analogen kodiert, in ein geeignetes Hefe-Expressions-Vehikel insertiert ist, das, wenn es in Hefe überführt wird, in der Lage ist, den Vorläufer des Insulin-Analogen zu exprimieren und zu sekretieren, in dem [Lys^{B28}], [Arg^{B28}], [Lys^{B29}] oder [Arg^{B29}] mit Gly^{A1} verbunden ist durch eine Peptidbindung oder ein Peptid der Formel III

-R(n)-R(1)-

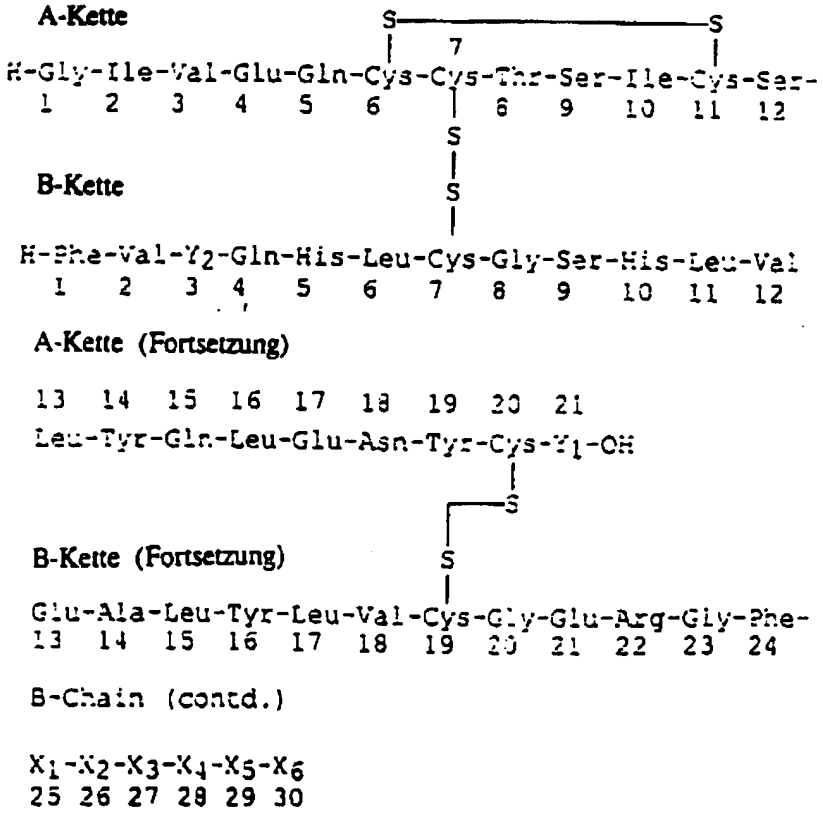
wobei R eine Peptidkette mit n Aminosäureresten, n eine ganze Zahl von 0 bis 33 und R(1) Lys oder Arg ist, der transformierte Hefestamm in einem geeigneten Nährmedium gezüchtet wird, und der Vorläufer aus dem Kulturmedium wiedergewonnen und mit einer Aminoverbindung der Formel IV

Q-QR'

umgesetzt wird, wobei Q ein einzelner Aminosäurerest ist und R^a eine Carboxy-Schutzgruppe wie Methyl oder tert-Butyl ist, unter Verwendung von Trypsin oder einem Trypsin-artigen Enzym als Katalysator in einem Gemisch von Wasser und organischen Lösungsmitteln, woraufhin die Carboxy-Schutzgruppe entfernt wird und das Insulin-Analoge aus dem Reaktionsgemisch isoliert wird, oder

ein Vorläufer eines Insulin-Analogen, in dem die C-terminale Aminosäure nicht Lys oder Arg ist, wobei der Vorläufer eine Brücke aus einem einzelnen Paar von basischen Aminosäuren, ausgewählt aus der Gruppe, bestehend aus Lys und Arg, zwischen dem C-terminalen und Gly^{A1} besitzt, isoliert werden kann und dann durch enzymatische Behandlung unter Verwendung von Trypsin oder Carboxypeptidase B in das Insulin-Analoge umgewandelt werden kann.

2. Verfahren zur Herstellung eines Analogen von menschlichem Insulin mit der folgenden Formel:



wobei X_1, X_2, X_3, Y_1 und Y_2 jede beliebige, natürlich vorkommende Aminosäure sind; X_4 Lys oder Arg ist; X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen, natürlich vorkommenden Aminosäure mit Ausnahme von Pro; und X_6 jede beliebige natürlich vorkommende Aminosäure ist, die die C-terminale Hydroxygruppe trägt, oder -OH oder X_5 und X_6 zusammen die C-terminale Hydroxygruppe bilden, wobei menschliches Des (B23-B30)-Insulin hergestellt wird durch Behandeln eines Insulins mit Trypsin unter Abspaltung der (B23-B30)-Aminosäuren, das gewünschte Peptid von sechs bis acht Aminosäuren synthetisiert wird, das resultierende Peptid an menschliches Des(B23-B30)-Insulin gekoppelt wird und das resultierende Insulin-Analoge aus dem Reaktionsgemisch isoliert wird.

3. Verfahren nach Anspruch 1 oder 2, wobei Y_1 und/oder Y_2 ausgewählt sind aus der Gruppe, bestehend aus jeder beliebigen, natürlich vorkommenden Aminosäure mit Ausnahme von Asn.

4. Verfahren nach Anspruch 1 oder 2, wobei

X_1 ausgewählt ist aus der Gruppe, bestehend aus Phe, Ala, His, Thr, Ser, Asn oder Tyr;
X_2 ausgewählt ist aus der Gruppe, bestehend aus Tyr, Thr, Glu, Asp, Ala, His, Ser oder Phe;
X_3 ausgewählt ist aus der Gruppe, bestehend aus Pro, Glu, Asp, Ser, Thr oder His;
X_5 ausgewählt ist aus der Gruppe, bestehend aus Lys, Thr, Ser, Ala, Asp oder Glu;
X_6 ausgewählt ist aus der Gruppe, bestehend aus Thr-OH, Ser-OH, Ala-OH, Asp-OH, Glu-OH oder -OH, oder X_5 und X_6 zusammen die C-terminale Hydroxygruppe bilden;
Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Gly, Ser, Glu oder Ala und
Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Glu oder Asp.

5. Verfahren nach Anspruch 1 oder 2, wobei

X_1 ausgewählt ist aus der Gruppe, bestehend aus Phe, Ala, His, Thr, Ser, Asn oder Tyr;
X_2 ausgewählt ist aus der Gruppe, bestehend aus Tyr, Thr, Glu, Asp, Ala, His, Ser oder Phe;
X_3 ausgewählt ist aus der Gruppe, bestehend aus Pro, Glu, Asp, Ser, Thr oder His;
X_5 ausgewählt ist aus der Gruppe, bestehend aus Lys, Thr, Ser, Ala, Asp oder Glu;
X_6 -OH ist;
Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Gly, Ser, Glu oder Ala und
Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Glu oder Asp.

6. Verfahren nach Anspruch 1 oder 2, wobei

X_1 ausgewählt ist aus der Gruppe, bestehend aus Phe, Ala, His, Thr, Ser, Asn oder Tyr;
X_2 ausgewählt ist aus der Gruppe, bestehend aus Tyr, Thr, Glu, Asp, Ala, His, Ser oder Phe;
X_3 ausgewählt ist aus der Gruppe, bestehend aus Pro, Glu, Asp, Ser, Thr oder His;
X_5 und X_6 zusammen die C-terminale Hydroxygruppe bilden;
Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Gly, Glu, Ser oder Ala und
Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Glu oder Asp.

7. Verfahren nach Anspruch 1 oder 2, wobei
X_1 Phe ist; X_2 Tyr ist; X_3 Thr ist; X_5 Lys ist; X_6 Thr-OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

8. Verfahren nach Anspruch 1 oder 2, wobei X_1 Tyr ist; X_2 Thr ist; X_3 Pro ist; X_5 Thr ist; X_6 -OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

9. Verfahren nach Anspruch 1 oder 2, wobei X_1 Phe ist; X_2 Thr ist; X_3 Pro ist; X_5 Thr ist; X_6 -OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

10. Verfahren nach Anspruch 1 oder 2, wobei X_1 Phe ist; X_2 Tyr ist; X_3 Pro ist; X_5 Thr ist; X_6 -OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

11. Verfahren nach Anspruch 1 oder 2, wobei die Aminosäure X_1 keine Ladung besitzt und ein Kohlenstoffatom in der Gamma-Position besitzt, das sp^2-hybridisiert ist und X_6 -OH ist.

12. Verfahren nach Anspruch 11, wobei Y_1 und Y_2 ausgewählt sind aus der Gruppe, bestehend aus jeder beliebigen natürlich vorkommenden Aminosäure mit Ausnahme von Asn und X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen natürlich vorkommenden Aminosäure mit Ausnahme von Thr und Pro.

13. Verfahren nach Anspruch 11, wobei X_5 und X_6 zusammen -OH bilden.

14. Verfahren zur Herstellung einer pharmazeutischen Zusammensetzung, umfassend das Formulieren eines Analogen von menschlichem Insulin, hergestellt durch das Verfahren nach einem der Ansprüche 1 bis 13 oder eines pharmazeutisch verträglichen Salzes davon mit einem pharmazeutisch verträglichen Träger.

15. Verfahren nach Anspruch 14, wobei das Insulin-Analoge eine geringe Löslichkeit bei pH 7,3 aufweist.

16. Verfahren nach Anspruch 15, wobei das Insulin-Analoge im wesentlichen monomer ist.

17. Verfahren nach Anspruch 14, wobei die pharmazeutische Zusammensetzung als eine wäßrige Lösung formuliert ist.

18. Verfahren nach Anspruch 14, wobei die pharmazeutische Zusammensetzung als eine wäßrige Suspension formuliert ist.

19. Verfahren nach Anspruch 14, wobei der pharmazeutisch verträgliche Träger eine wäßrige isotonische Lösung ist.

20. Verfahren nach Anspruch 17, 18 oder 19, wobei die wäßrige Lösung, wäßrige Suspension oder wäßrige isotonische Lösung weiterhin Zinkionen und/oder einen Puffer umfaßt wie Acetat oder Citrat und/oder einen Konservierungsstoff wie m-Kresol, Methylparaben oder Phenol.

21. Verfahren nach Anspruch 14, wobei die pharmazeutische Zusammensetzung mehr als ein Insulin-Analoges umfaßt.

22. Verfahren nach Anspruch 14, wobei die pharmazeutische Zusammensetzung für eine mukosale oder transkutane Verabreichung formuliert ist.

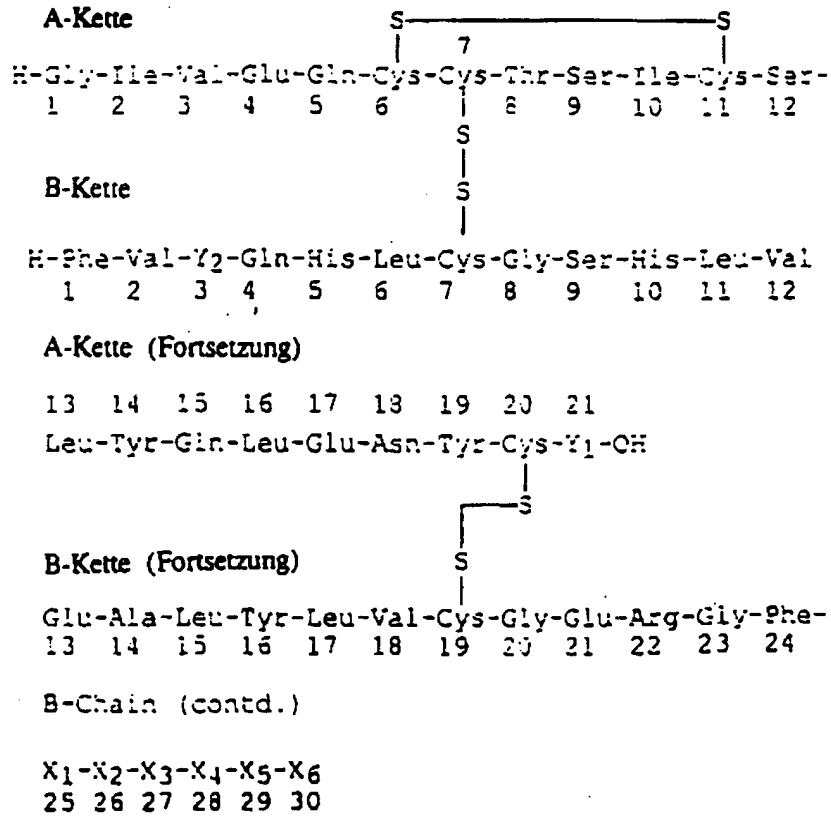
23. Verfahren nach Anspruch 14, wobei die pharmazeutische Zusammensetzung für eine parenterale Verabreichung formuliert ist.

24. Verwendung einer Verbindung, die durch das Verfahren nach einem der Ansprüche 1 bis 13 hergestellt wurde, zur Herstellung eines Medikaments zur Verwendung bei der Behandlung von Diabetes.

Patentansprüche für folgenden Vertragsstaat : GR

1. Analoge von menschlichem Insulin, gekennzeichnet dadurch, daß sie einen positiv geladenen Aminosäurerest, d.h. Lys oder Arg, an Position B29 besitzen, d.h. in Position 8 in der B-Kette, gerechnet von [Gly^{B20}], daß sie wahlweise zusätzlich in dem C-terminalen Ende der B-Kette von [Phe^{B24}] bis zu dem C-terminalen Aminosäurerest modifiziert sind, mit der Maßgabe, daß sich kein Pro in Position B29 befindet, und daß wahlweise A21 und/oder B3 nicht Asn sind.

2. Analoge von menschlichem Insulin, dadurch gekennzeichnet, daß sie die folgende Formel besitzen:



wobei X_1, X_2, X_3, Y_1 und Y_2 jede beliebige, natürlich vorkommende Aminosäure sind; X_4 Lys oder Arg ist; X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen, natürlich vorkommenden Aminosäure mit Ausnahme von Pro; und X_6 jede beliebige natürlich vorkommende Aminosäure ist, die die C-terminale Hydroxygruppe trägt, oder -OH oder X_5 und X_6 zusammen die C-terminale Hydroxygruppe bilden.

3. Analoge von menschlichem Insulin nach Anspruch 2, wobei Y_1 und/ oder Y_2 ausgewählt sind aus der Gruppe, bestehend aus jeder beliebigen, natürlich vorkommenden Aminosäure mit Ausnahme von Asn.

4. Analoge von menschlichem Insulin nach Anspruch 2, wobei

   X_1 ausgewählt ist aus der Gruppe, bestehend aus Phe, Ala, His, Thr, Ser, Asn oder Tyr;
   X_2 ausgewählt ist aus der Gruppe, bestehend aus Tyr, Thr, Glu, Asp, Ala, His, Ser oder Phe;
   X_3 ausgewählt ist aus der Gruppe, bestehend aus Pro, Glu, Asp, Ser, Thr oder His;
   X_5 ausgewählt ist aus der Gruppe, bestehend aus Lys, Thr, Ser, Ala, Asp oder Glu;
   X_6 ausgewählt ist aus der Gruppe, bestehend aus Thr-OH, Ser-OH, Ala-OH, Asp-OH, Glu-OH oder -OH, oder X_5 und X_6 zusammen die C-terminale Hydroxygruppe bilden;
   Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Gly, Ser, Glu oder Ala und
   Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Glu oder Asp.

5. Analoge von menschlichem Insulin nach Anspruch 2, wobei

   X_1 ausgewählt ist aus der Gruppe, bestehend aus Phe, Ala, His, Thr, Ser, Asn oder Tyr;
   X_2 ausgewählt ist aus der Gruppe, bestehend aus Tyr, Thr, Glu, Asp, Ala, His, Ser oder Phe;
   X_3 ausgewählt ist aus der Gruppe, bestehend aus Pro, Glu, Asp, Ser, Thr oder His;
   X_5 ausgewählt ist aus der Gruppe, bestehend aus Lys, Thr, Ser, Ala, Asp oder Glu;

X_6 -OH ist;
Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Gly, Ser, Glu oder Ala und
Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Glu oder Asp.

6. Analoge von menschlichem Insulin nach Anspruch 2, wobei

X_1	ausgewählt ist aus der Gruppe, bestehend aus Phe, Ala, His, Thr, Ser, Asn oder Tyr;
X_2	ausgewählt ist aus der Gruppe, bestehend aus Tyr, Thr, Glu, Asp, Ala, His, Ser oder Phe;
X_3	ausgewählt ist aus der Gruppe, bestehend aus Pro, Glu, Asp, Ser, Thr oder His;
X_5 und X_6	zusammen die C-terminale Hydroxygruppe bilden;
Y_1	ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Gly, Glu, Ser oder Ala und
Y_2	ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Glu oder Asp.

7. Analoge von menschlichem Insulin nach Anspruch 2, wobei X_1 Phe ist; X_2 Tyr ist; X_3 Thr ist; X_5 Lys ist; X_6 Thr-OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

8. Analoge von menschlichem Insulin nach Anspruch 2, wobei X_1 Tyr ist; X_2 Thr ist; X_3 Pro ist; X_5 Thr ist; X_6 -OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

9. Analoge von menschlichem Insulin nach Anspruch 2, wobei X_1 Phe ist; X_2 Thr ist; X_3 Pro ist; X_5 Thr ist; X_6 -OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

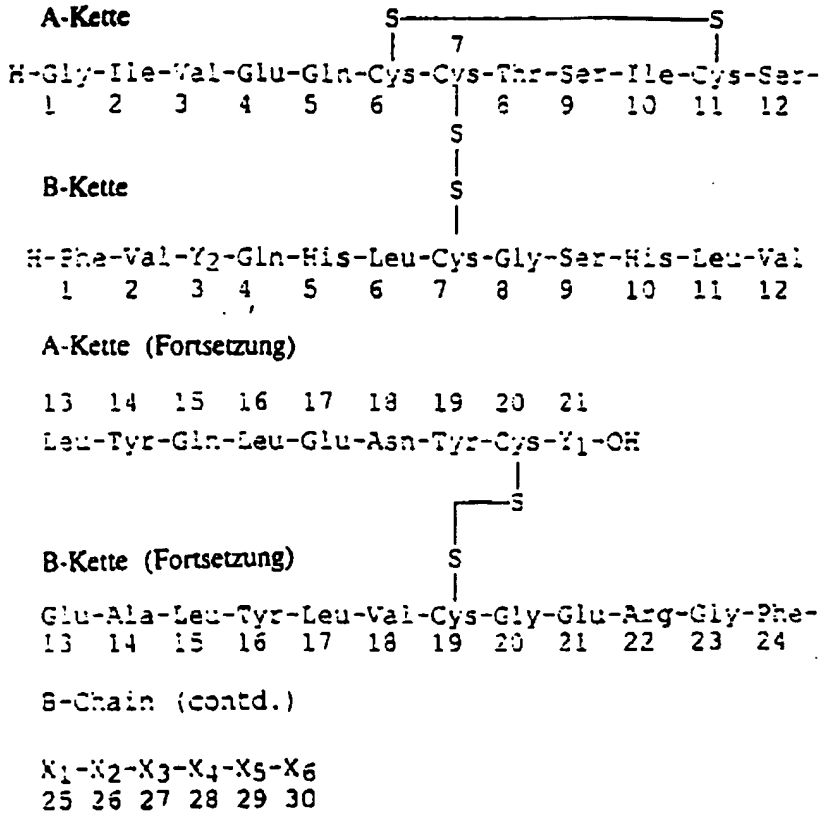
10. Analoge von menschlichem Insulin nach Anspruch 2, wobei X_1 Phe ist; X_2 Tyr ist; X_3 Pro ist; X_5 Thr ist; X_6 -OH ist; Y_1 ausgewählt ist aus der Gruppe, bestehend aus Asn, Asp, Ser oder Gly und Y_2 ausgewählt ist aus der Gruppe, bestehend aus Asn, Gln, Asp oder Glu.

11. Analoge von menschlichem Insulin nach Anspruch 2, wobei die Aminosäure X_1 keine Ladung besitzt und ein Kohlenstoffatom in der Gamma-Position besitzt, das sp^2-hybridisiert ist und X_6 -OH ist.

12. Analoge von menschlichem Insulin nach Anspruch 11, wobei Y_1 und Y_2 ausgewählt sind aus der Gruppe, bestehend aus jeder beliebigen natürlich vorkommenden Aminosäure mit Ausnahme von Asn und X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen natürlich vorkommenden Aminosäure mit Ausnahme von Thr und Pro.

13. Analoge von menschlichem Insulin nach Anspruch 11, wobei X_5 und X_6 zusammen -OH bilden.

14. Verfahren zur Herstellung eines Analogen von menschlichem Insulin mit der folgenden Formel:



wobei X_1, X_2, X_3, Y_1 und Y_2 jede beliebige, natürlich vorkommende Aminosäure sind: X_4 Lys oder Arg ist; X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen, natürlich vorkommenden Aminosäure mit Ausnahme von Pro; und X_6 jede beliebige natürlich vorkommende Aminosäure ist, die die C-terminale Hydroxygruppe trägt, oder -OH oder X_5 und X_6 zusammen die C-terminale Hydroxygruppe bilden, wobei eine DNA-Sequenz, die ein Vorläufermolekül des Insulin-Analogen kodiert, in ein geeignetes Hefe-Expressions-Vehikel insertiert ist, das, wenn es in Hefe überführt wird, in der Lage ist, den Vorläufer des Insulin-Analogen zu exprimieren und zu sekretieren, in dem [$LysB^{28}$], [Arg^{B28}], [Lys^{B29}] oder [Arg^{B29}] mit Gly^{A1} verbunden ist durch eine Peptidbindung oder ein Peptid der Formel III

-R(n)-R(1)-

wobei R eine Peptidkette mit n Aminosäureresten, n eine ganze Zahl von 0 bis 33 und R(1) Lys oder Arg ist, der transformierte Hefestamm in einem geeigneten Nährmedium gezüchtet wird, und der Vorläufer aus dem Kulturmedium wiedergewonnen und mit einer Aminoverbindung der Formel IV

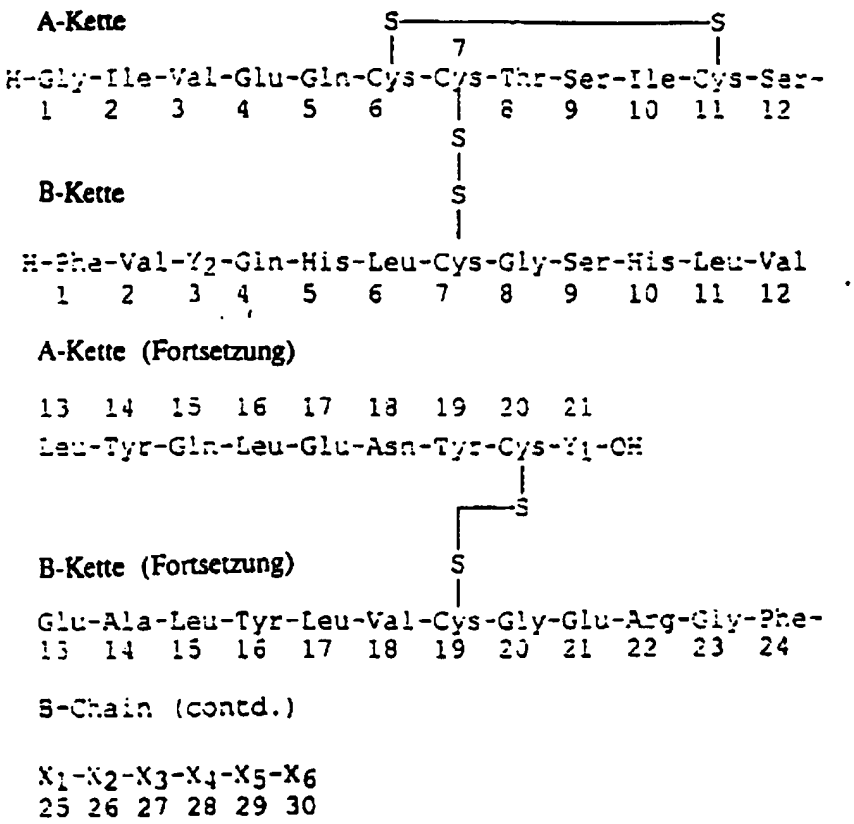
Q-QR"

umgesetzt wird, wobei Q ein einzelner Aminosäurerest ist und R" eine Carboxy-Schutzgruppe wie Methyl oder tert-Butyl ist, unter Verwendung von Trypsin oder einem Trypsin-artigen Enzym als Katalysator in einem Gemisch von Wasser und organischen Lösungsmitteln, woraufhin die Carboxy-Schutzgruppe entfernt wird und das Insulin-Analoge aus dem Reaktionsgemisch isoliert wird, oder

ein Vorläufer eines Insulin-Analogen, in dem die C-terminale Aminosäure nicht Lys oder Arg ist, wobei der Vorläufer eine Brücke aus einem einzelnen Paar von basischen Aminosäuren, ausgewählt aus der Gruppe, bestehend aus Lys und Arg, zwischen dem C-terminalen und Gly^{A1} besitzt, isoliert werden kann und dann durch enzymatische

Behandlung unter Verwendung von Trypsin oder Carboxypeptidase B in das Insulin-Analoge umgewandelt werden kann.

15. Verfahren zur Herstellung eines Analogen von menschlichem Insulin mit der folgenden Formel:



wobei X_1, X_2, X_3, Y_1 und Y_2 jede beliebige, natürlich vorkommende Aminosäure sind; X_4 Lys oder Arg ist; X_5 ausgewählt ist aus der Gruppe, bestehend aus jeder beliebigen, natürlich vorkommenden Aminosäure mit Ausnahme von Pro; und X_6 jede beliebige natürlich vorkommende Aminosäure ist, die die C-terminale Hydroxygruppe trägt, oder -OH oder X_5 und X_6 zusammen die C-terminale Hydroxygruppe bilden, wobei menschliches Des(B23-B30)-Insulin hergestellt wird durch Behandeln eines Insulins mit Trypsin unter Abspaltung der (B23-B30)-Aminosäuren, das gewünschte Peptid von sechs bis acht Aminosäuren synthetisiert wird, das resultierende Peptid an menschliches Des-(B23-B30)Insulin gekoppelt wird und das resultierende Insulin-Analoge aus dem Reaktionsgemisch isoliert wird.

16. Verfahren zur Herstellung einer pharmazeutischen Zusammensetzung, umfassend das Formulieren eines Analogen von menschlichem Insulin gemäß einem der Ansprüche 1 bis 13 oder eines pharmazeutisch verträglichen Salzes davon mit einem pharmazeutisch verträglichen Träger.

17. Verfahren nach Anspruch 16, wobei das Insulin-Analoge eine geringe Löslichkeit bei pH 7,3 aufweist.

18. Verfahren nach Anspruch 17, wobei das Insulin-Analoge im wesentlichen monomer ist.

19. Verfahren nach Anspruch 16, wobei die pharmazeutische Zusammensetzung als eine wäßrige Lösung formuliert ist.

20. Verfahren nach Anspruch 16, wobei die pharmazeutische Zusammensetzung als eine wäßrige Suspension for-

muliert ist.

21. Verfahren nach Anspruch 16, wobei der pharmazeutisch verträgliche Träger eine wäßrige isotonische Lösung ist.

22. Verfahren nach Anspruch 19, 20 oder 21, wobei die wäßrige Lösung, wäßrige Suspension oder wäßrige isotonisch Lösung weiterhin Zinkionen und/oder einen Puffer umfaßt wie Acetat oder Citrat und/oder einen Konservierungsstoff wie m-Kresol, Methylparaben oder Phenol.

23. Verfahren nach Anspruch 16, wobei die pharmazeutische Zusammensetzung mehr als ein Insulin-Analoges umfaßt.

24. Verfahren nach Anspruch 16, wobei die pharmazeutische Zusammensetzung für eine mukosale oder transkutane Verabreichung formuliert ist.

25. Verfahren nach Anspruch 16, wobei die pharmazeutische Zusammensetzung für eine parenterale Verabreichung formuliert ist.

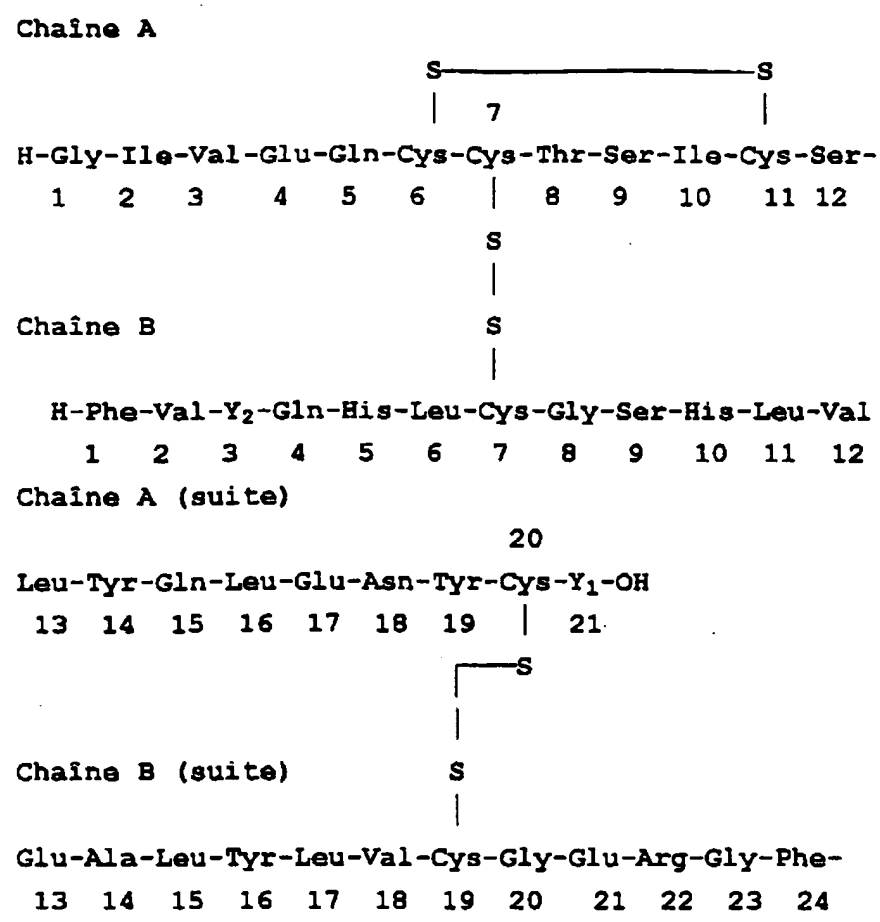
26. Verwendung einer Verbindung gemäß einem der Ansprüche 1 bis 13 zur Herstellung eines Medikaments zur Verwendung bei der Behandlung von Diabetes.

Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, DE, FR, GB, IT, LI, LU, NL, SE

1. Analogues d'insuline humaine, caractérisés en ce qu'ils ont un résidu d'acide aminé chargé positivement, c'est-à-dire Lys ou Arg, à la position B28, c'est-à-dire à la position 8 dans la chaîne B calculée à partir de [Gly^{B20}], qu'ils sont en option de plus modifiés à l'extrémité C-terminale de la chaîne B de [Phe^{B24}] au résidu d'acide aminé C-terminal, à la condition qu'il n'y ait pas Pro à la Position B29, et qu'en option A21 et/ou B3 sont différents de Asn.

2. Analogues d'insuline humaine, caractérisés en ce qu'ils ont la formule :



Chaîne B (suite)

X_1-X_2-X_3-X_4-X_5-X_6

25 26 27 28 29 30

où X_1, X_2, X_3, Y_1 et Y_2 sont tout résidu d'acide aminé survenant naturellement, X_4 est Lys ou Arg, X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Pro, et X_6 est tout résidu d'acide aminé survenant naturellement portant le groupe hydroxyle, ou -OH, de l'extrémité C-terminale, ou X_5 et X_6 forment ensemble le groupe hydroxyle de l'extrémité C-terminale.

3. Analogues d'insuline humaine selon la revendication 2, dans lesquels Y_1 et/ou Y_2 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Asn.

4. Analogues d'insuline humaine selon la revendication 2, dans lesquels

- X_1 est sélectionné parmi le groupe constitué de Phe, Ala, His, Thr, Ser, Asn ou Tyr,
- X_2 est sélectionné parmi le groupe constitué de Tyr, Thr, Glu, Asp, Ala, His, Ser ou Phe,
- X_3 est sélectionné parmi le groupe constitué de Pro, Glu, Asp, Ser, Thr ou His,
- X_5 est sélectionné parmi le groupe constitué de Lys, Thr, Ser, Ala, Asp ou Glu,
- X_6 est sélectionné parmi le groupe constitué de Thr-OH, Ser-OH, Ma-OH, Asp-OH, Glu-OH ou -OH, ou X_5 et X_6 forment ensemble le groupe hydroxyle de l'extrémité C-terminale,
- Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Gly, Ser, Glu ou Ala et

Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Glu ou Asp.

5. Analogues d'insuline humaine selon la revendication 2, dans lesquels

X_1 est sélectionné parmi le groupe constitué de Phe, Ala, His, Thr, Ser, Asn ou Tyr,
X_2 est sélectionné parmi le groupe constitué de Tyr, Thr, Glu, Asp, Ala, His, Ser ou Phe,
X_3 est sélectionné parmi le groupe constitué de Pro, Glu, Asp, Ser, Thr ou His,
X_5 est sélectionné parmi le groupe constitué de Lys, Thr, Ser, Ala, Asp ou Glu,
X_6 est -OH,
Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Gly, Ser, Glu ou Ala et
Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Glu ou Asp.

6. Analogues d'insuline humaine selon la revendication 2, dans lesquels

X_1	est sélectionné parmi le groupe constitué de Phe, Ala, His, Thr, Ser, Asn ou Tyr,
X_2	est sélectionné parmi le groupe constitué de Tyr, Thr, Glu, Asp, Ala, His, Ser ou Phe,
X_3	est sélectionné parmi le groupe constitué de Pro, Glu, Asp, Ser, Thr ou His,
X_5 et X_6	forment ensemble le groupe hydroxyle de la terminaison C-terminale,
Y_1	est sélectionné parmi le groupe constitué de Asn, Asp, Gly, Glu, Ser ou Ala, et
Y_2	est sélectionné parmi le groupe constitué de Asn, Gln, Glu ou Asp.

7. Analogues d'insuline humaine selon la revendication 2, dans lesquels X_1 est Phe, X_2 est Tyr, X_3 est Thr, X_5 est Lys, X_6 est Thr-OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

8. Analogues d'insuline humaine selon la revendication 2, dans lesquels X_1 est Tyr, X_2 est Thr, X_3 est Pro, X_5 est Thr, X_6 est -OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

9. Analogues d'insuline humaine selon la revendication 2, dans lesquels X_1 est Phe, X_2 est Thr, X_3 est Pro, X_5 est Thr, X_6 est -OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

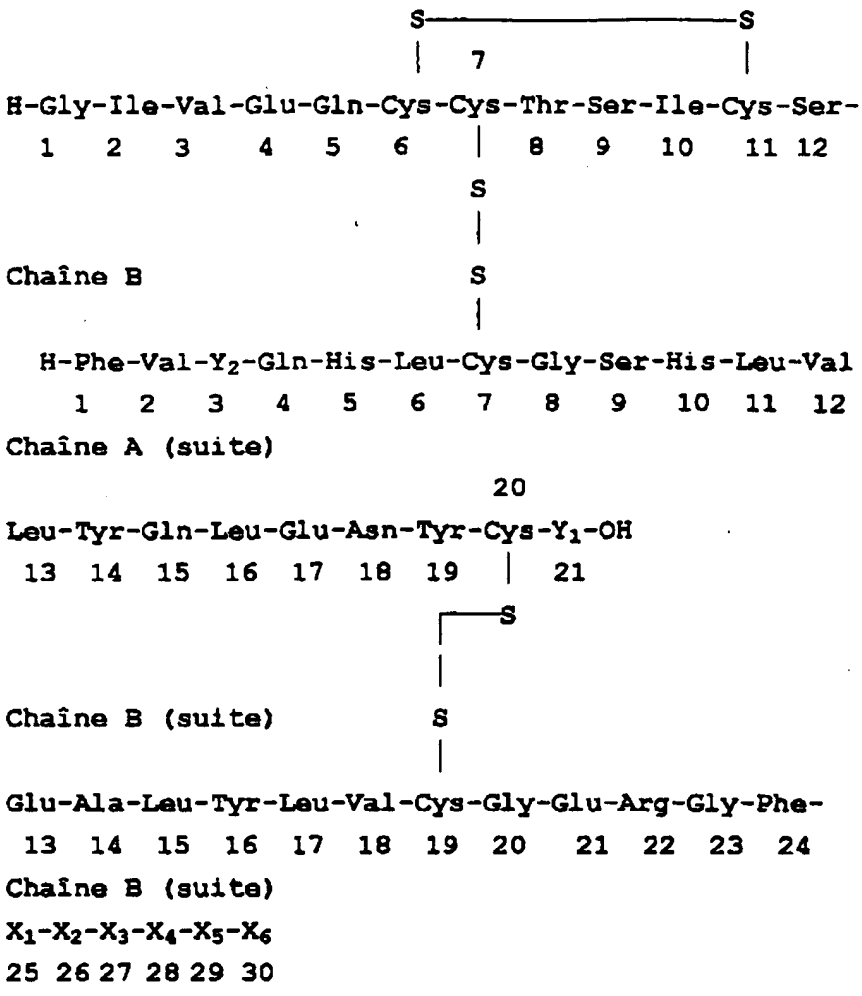
10. Analogues d'insuline humaine selon la revendication 2, dans lesquels X_1 est Phe, X_2 est Tyr, X_3 est Pro, X_5 est Thr, X_6 est -OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

11. Analogues d'insuline humaine selon la revendication 2, dans lesquels ledit acide aminé X_1 est non-chargé et a un atome de carbone à la position gamma qui est hybridé par sp^2 et X_6 est -OH.

12. Analogues d'insuline humaine selon la revendication 11, dans lesquels Y_1 et Y_2 sont sélectionnés parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Asn et X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Thr et Pro.

13. Analogues d'insuline humaine selon la revendication 11, dans lesquels X_5 et X_6 forment ensemble -OH.

14. Composition pharmaceutique comportant un analogue d'insuline humaine ayant la formule suivante : Chaîne A



où X_1, X_2, X_3, Y_1 et Y_2 sont tout résidu d'acide aminé survenant naturellement, X_4 est Lys ou Arg, X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Pro, et X_6 est tout résidu d'acide aminé survenant naturellement portant le groupe hydroxyle, ou -OH, de l'extrémité C-terminale, ou X_5 et X_6 forment ensemble le groupe hydroxyle de l'extrémité C-terminale ou un sel de celui-ci pouvant être accepté pharmaceutiquement et un support pouvant être accepté pharmaceutiquement.

15. Composition pharmaceutique selon la revendication 14, dans laquelle ledit analogue d'insuline présente une faible solubilité à pH 7,3.

16. Composition pharmaceutique selon la revendication 15, dans laquelle ledit analogue d'insuline est essentiellement monomérique.

17. Composition pharmaceutique selon la revendication 14, dans laquelle ladite composition pharmaceutique est formulée sous la forme d'une solution aqueuse.

18. Composition pharmaceutique selon la revendication 14, dans laquelle ladite composition pharmaceutique est formulée sous la forme d'une suspension aqueuse.

19. Composition pharmaceutique selon la revendication 14, dans laquelle ledit support pouvant être accepté pharmaceutiquement est une solution isotonique, aqueuse.

20. Composition pharmaceutique selon la revendication 17, 18 ou 19, dans laquelle lesdites solution aqueuse, suspension aqueuse ou solution isotonique aqueuse comportent de plus des ions zinc et/ou une solution tampon, telle que de l'acétate ou du citrate et/ou un agent de conservation tel que du m-crésol, du p-hydroxybenzoate de méthyle ou du phénol.

21. Composition pharmaceutique selon la revendication 14, dans laquelle ladite composition pharmaceutique comporte plus de un analogue d'insuline.

22. Composition pharmaceutique selon la revendication 14, dans laquelle ladite composition pharmaceutique est formulée pour une administration par muqueuse ou transcutanée.

23. Composition pharmaceutique selon la revendication 14, dans laquelle une composition pharmaceutique est formulée pour une administration parentérale.

24. Composition pharmaceutique selon la revendication 14, dans laquelle X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Pro.

25. Composition pharmaceutique selon la revendication 14 ou 24, dans laquelle Y_1 et/ou Y_2 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Asn.

26. Composition pharmaceutique selon la revendication 14, dans laquelle

X_1 est sélectionné parmi le groupe constitué de Phe, Ala, His, Thr, Ser, Asn ou Tyr,
X_2 est sélectionné parmi le groupe constitué de Tyr, Thr, Glu, Asp, Ala, His, Ser ou Phe,
X_3 est sélectionné parmi le groupe constitué de Pro, Glu, Asp, Ser, Thr ou His,
X_5 est sélectionné parmi le groupe constitué de Lys, Thr, Ser, Ala, Asp ou Glu,
X_6 est sélectionné parmi le groupe constitué de Thr-OH, Ser-OH, Ma-OH, Asp-OH, Glu-OH ou -OH, ou X_5 et X_6 forment ensemble le groupe hydroxyle de l'extrémité C-terminale,
Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Gly, Ser, Glu ou Ala et
Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Glu ou Asp.

27. Composition pharmaceutique selon la revendication 14, dans laquelle

X_1 est sélectionné parmi le groupe constitué de Phe, Ala, His, Thr, Ser, Asn ou Tyr,
X_2 est sélectionné parmi le groupe constitué de Tyr, Thr, Glu, Asp, Ala, His, Ser ou Phe,
X_3 est sélectionné parmi le groupe constitué de Pro, Glu, Asp, Ser, Thr ou His,
X_5 est sélectionné parmi le groupe constitué de Lys, Thr, Ser, Ala, Asp ou Glu,
X_6 est -OH,
Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Gly, Ser, Glu ou Ala et
Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Glu ou Asp.

28. Composition pharmaceutique selon la revendication 14, dans laquelle

X_1 est sélectionné parmi le groupe constitué de Phe, Ala, His, Thr, Ser, Asn ou Tyr,
X_2 est sélectionné parmi le groupe constitué de Tyr, Thr, Glu, Asp, Ala, His, Ser ou Phe,
X_3 est sélectionné parmi le groupe constitué de Pro, Glu, Asp, Ser, Thr ou His,
X_5 et X_6 forment ensemble le groupe hydroxyle de la terminaison C-terminale,
Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Gly, Glu, Ser ou Ala, et
Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Glu ou Asp.

29. Composition pharmaceutique selon la revendication 14, dans laquelle X_1 est Phe, X_2 est Tyr, X_3 est Thr, X_5 est Lys, X_6 est Thr-OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

30. Composition pharmaceutique selon la revendication 14, dans laquelle X_1 est Tyr, X_2 est Thr, X_3 est Pro, X_5 est Thr, X_6 est -OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

31. Composition pharmaceutique selon la revendication 14, dans laquelle X_1 est Phe, X_2 est Thr, X_3 est Pro, X_5 est Thr, X_6 est -OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

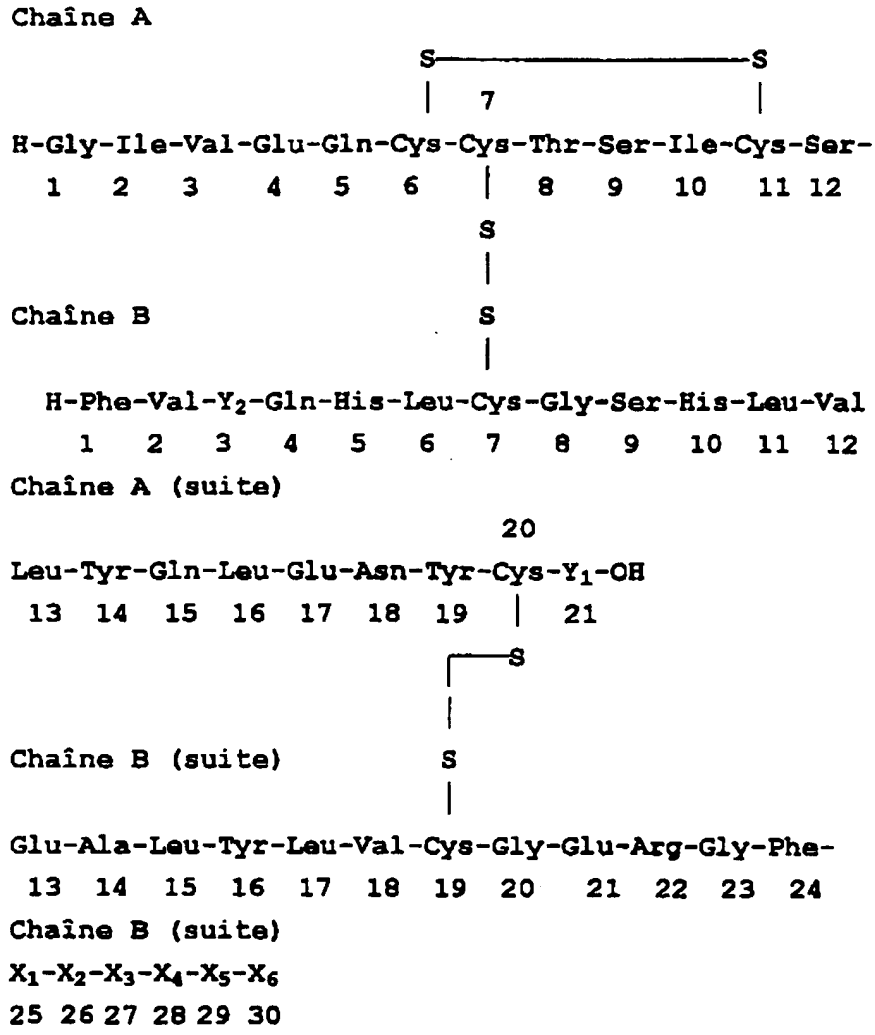
32. Composition pharmaceutique selon la revendicati n 14, dans laquelle X_1 est Phe, X_2 est Tyr, X_3 est Pro, X_5 est Thr, X_6 est -OH, yl est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

33. Composition pharmaceutique selon la revendication 14, dans laquelle ledit acide aminé X_1 est non-chargé et a un atome de carbone à la position gamma qui est hybridé par sp^2 et X_6 est -OH.

34. Composition pharmaceutique selon la revendication 33, dans laquelle Y_1 et Y_2 sont sélectionnés parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Asn et X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Thr.

35. Composition pharmaceutique selon la revendication 33, dans laquelle X_5 et X_6 forment ensemble -OH.

36. Processus pour préparer un analogue d'insuline humaine ayant la formule suivante :



où X_1, X_2, X_3, Y_1 et Y_2 sont tout résidu d'acide aminé survenant naturellement, X_4 est Lys ou Arg, X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Pro, et X_6 est tout résidu d'acide aminé survenant naturellement portant le groupe hydroxyle, ou -OH, de l'extrémité C-terminal , u X_5 et X_6 forment ensemble le groupe hydroxyle d l'extrémité c-terminale, une séquence d'ADN codant pour un précurseur de l'analogue d'insuline en question étant inséré dans un véhicule d'expression de levure adapté qui, lorsqu'il est transféré dans la levure, est capable d'exprimer et de sécréter le précurseur de l'analogue d'insuline dans lequel [Lys^{B28}], [Arg^{B28}], [Lys^{B29}] ou [Arg^{B29}] est relié à Gly^{A1} par une liaison peptidique ou un peptide ayant la formule III

$$-R_n-R^1-$$

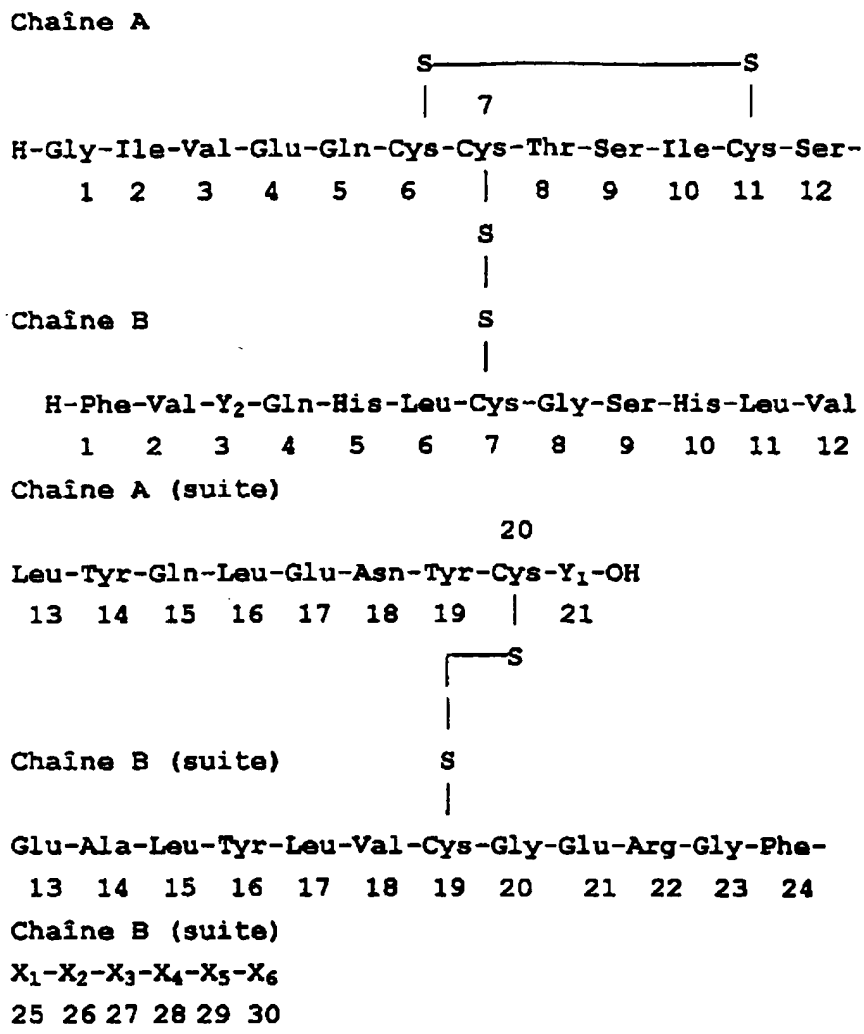
où R est une chaîne peptidique ayant n résidus d'acide aminé, n est un entier compris entre 0 et 33 et R^1 est Lys ou Arg, la souche de levure transformée est cultivée dans un milieu nutritif adapté, et le précurseur est récupéré à partir du bouillon de culture et mis à réagir avec un composé aminé ayant la formule IV

$$Q\text{-}OR'$$

Où Q est un résidu d'acide aminé unique et R' est un groupe de protection carboxyle tel qu'un méthyle ou un tert-butyle, en utilisant de la trypsine ou une enzyme du type trypsine en tant que catalyseur dans un mélange d'eau et de solvants organiques, après ceci le groupe de protection carboxyle est éliminé et l'analogue d'insuline est isolé du mélange de réaction, ou

un précurseur d'analogue d'insuline dans lequel l'acide aminé de l'extrémité C-terminale est différent de Lys ou de Arg, ledit précurseur ayant un pont constitué d'une paire unique d'acides aminés basiques sélectionnés parmi le groupe constitué de Lys et Arg entre l'extrémité C-terminale et Gly^{A1}peut être isolé et ensuite converti dans l'analogue d'insuline par traitement enzymatique en utilisant de la trypsine et une carboxypeptidase B.

37. Processus pour préparer un analogue d'insuline humaine ayant la formule suivante :

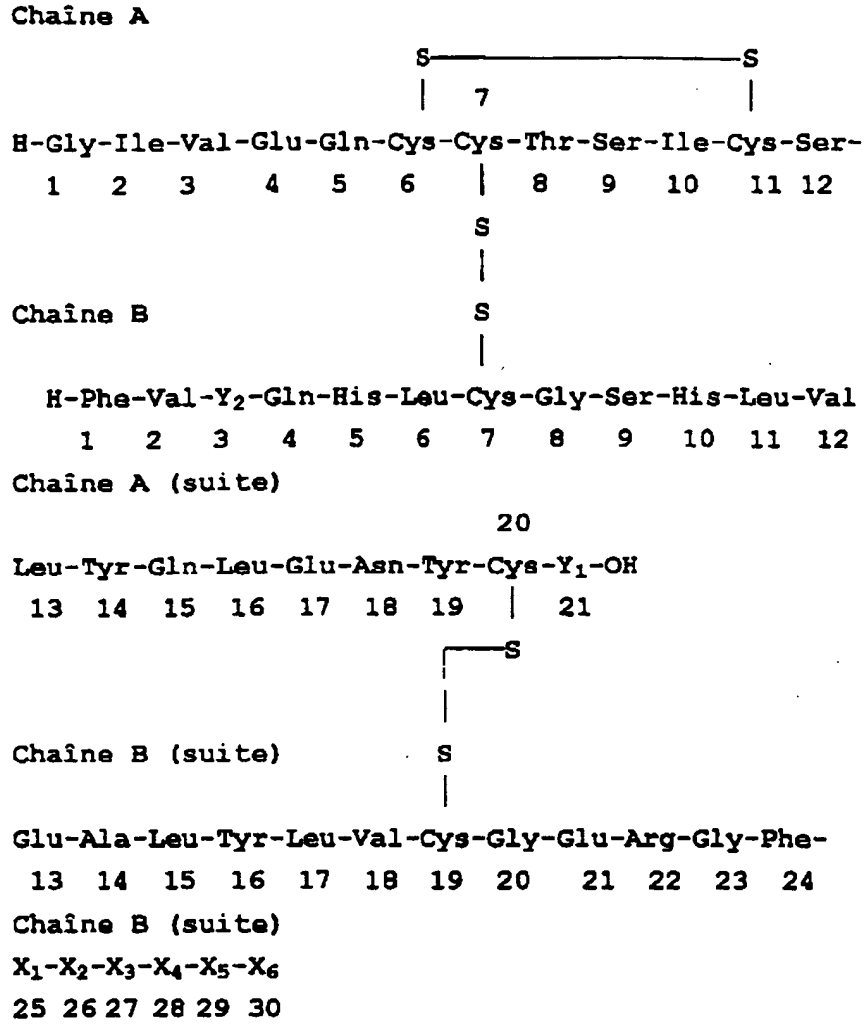


où X_1, X_2, X_3, Y_1 et Y_2 sont tout résidu d'acide aminé survenant naturellement, X_4 est Lys ou Arg, X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Pro, et X_6 est tout résidu d'acide aminé survenant naturellement portant le groupe hydroxyle, ou -OH, de l'extrémité C-terminale, ou X_5 et X_6 forment ensemble le groupe hydroxyle de l'extrémité C-terminale, dans lequel des(B23-B30)-insuline humaine est préparé en traitant une insuline par la trypsine pour cliver les acides aminés (B23 à B30), le peptide voulu de six à huit acides aminés est synthétisé, le peptide résultant est relié à des(B23-B30)-insuline humaine, et l'analogue d'insuline résultant est isolé du mélange de réaction.

38. Utilisation d'un composé selon l'une quelconque des revendications 1 à 13 pour la préparation d'un médicament destiné à être utilisé dans le traitement de diabètes.

Revendications pour l'Etat contractant suivant : ES

1. Processus pour préparer un analogue d'insuline humaine ayant la formule suivante :



où X_1, X_2, X_3, Y_1 et Y_2 sont tout résidu d'acide aminé survenant naturellement, X_4 est Lys ou Arg, X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Pro, et X_6 est tout résidu d'acide aminé survenant naturellement portant le groupe hydroxyle, ou -OH, de l'extrémité C-terminale, ou X_5 et X_6 forment ensemble le groupe hydroxyle de l'extrémité C-terminale, une séquence d'ADN codant pour un précurseur de l'analogue d'insuline en question étant inséré dans un véhicule d'expression de levure adapté qui, lorsqu'il est transféré dans la levure, est capable d'exprimer et de sécréter le précurseur de l'analogue d'insuline dans lequel [Lys^{B28}], [Arg^{B28}], [Lys^{B29}] ou [Arg^{B29}] est relié à Gly^{A1} par une liaison peptidique ou un peptide ayant la formule III

$$-R_n-R^1-$$

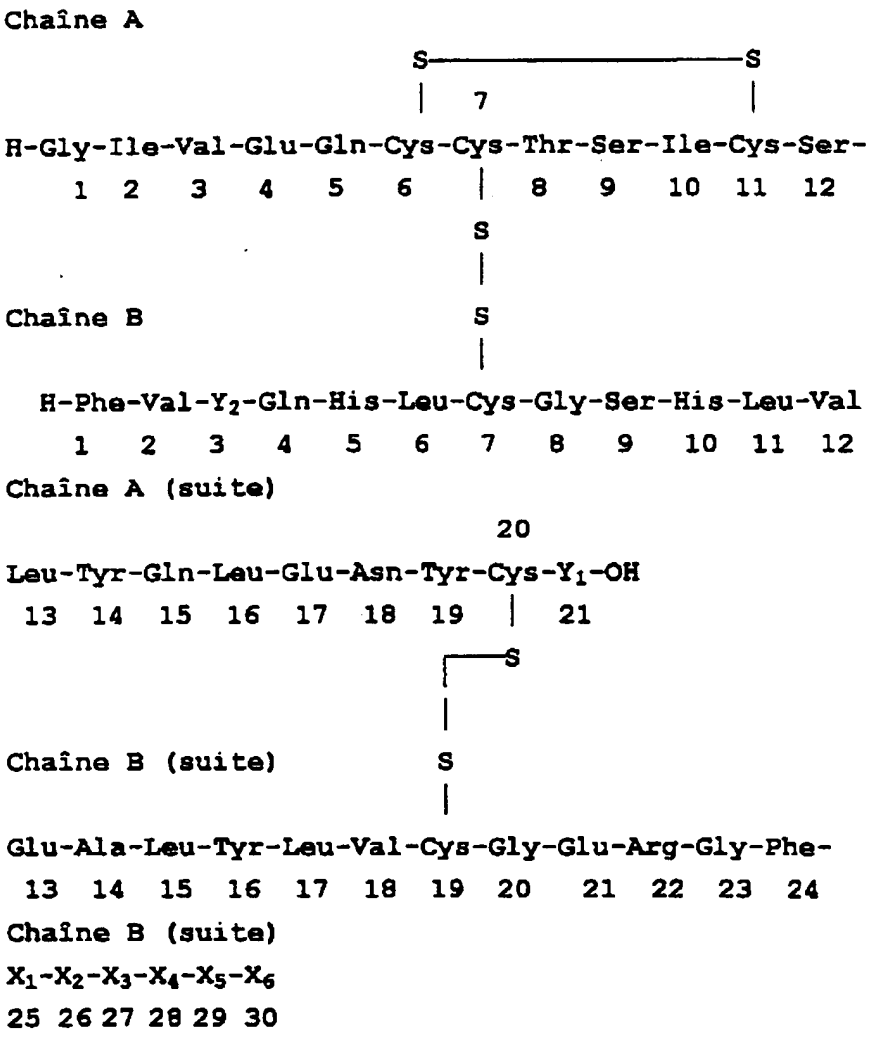
où R est une chaîne peptidique ayant n résidus d'acide aminé, n est un entier compris entre 0 et 33 et R^1 est Lys ou Arg, la souche de levure transformée est cultivée dans un milieu nutritif adapté, et le précurseur est récupéré à partir du bouillon de culture et mis à réagir avec un composé aminé ayant la formule IV

$$Q\text{-}OR'$$

Où Q est un résidu d'acide aminé unique et R" est un groupe de protection carboxyle tel qu'un méthyle ou un tert-butyle, en utilisant de la trypsine ou une enzyme du type trypsine en tant que catalyseur dans un mélange d'eau et de solvants organiques, après ceci le groupe de protection carboxyle est éliminé et l'analogue d'insuline est isolé du mélange d réaction, ou

un précurseur d'analogue d'insuline dans lequel l'acide aminé de l'extrémité C-terminale est différent de Lys ou de Arg, ledit précurseur ayant un pont constitué d'une paire unique d'acides aminés basiques sélectionnés parmi le groupe constitué de Lys et Arg entre l'extrémité C-terminale et Gly^{A1} peut être isolé et ensuite converti dans l'analogue d'insuline par traitement enzymatique en utilisant de la trypsine et une carboxypeptidase B.

2. Processus pour préparer un analogue d'insuline humaine ayant la formule suivante :



où X_1, X_2, X_3, Y_1 et Y_2 sont tout résidu d'acide aminé survenant naturellement, X_4 est Lys ou Arg, X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Pro, et X_6 est tout résidu d'acide aminé survenant naturellement portant le groupe hydroxyle, ou -OH, de l'extrémité C-terminale, ou X_5 et X_6 forment ensemble le groupe hydroxyle de l'extrémité C-terminale, dans lequel des(B23-B30)-insuline humaine est préparé en traitant une insuline par la trypsine pour cliver les acides aminés (B23 à B30), le peptide voulu de six à huit acides aminés est synthétisé, le peptide résultant est relié à des(B23-B30)-insuline humaine, et l'analogue d'insuline résultant est isolé du mélange de réaction.

3. Processus selon la revendication 1 ou 2, dans lequel Y_1 et/ou Y_2 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Asn.

4. Processus selon la revendication 1 ou 2, dans lequel

X_1 est sélectionné parmi le groupe constitué de Phe, Ala, His, Thr, Ser, Asn ou Tyr,
X_2 est sélectionné parmi le groupe constitué de Tyr, Thr, Glu, Asp, Ala, His, Ser ou Phe,
X_3 est sélectionné parmi le groupe constitué de Pro, Glu, Asp, Ser, Thr ou His,
X_5 est sélectionné parmi le groupe constitué de Lys, Thr, Ser, Ala, Asp ou Glu,
X_6 est sélectionné parmi le groupe constitué de Thr-OH, Ser-OH, Ala-OH, Asp-OH, Glu-OH ou -OH, ou X_5 et X_6 forment ensemble le groupe hydroxyle de l'extrémité C-terminale,
Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Gly, Ser, Glu ou Ala et
Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Glu ou Asp.

5. Processus selon la revendication 1 ou 2, dans lequel

X_1 est sélectionné parmi le groupe constitué de Phe, Ala, His, Thr, Ser, Asn ou Tyr,
X_2 est sélectionné parmi le groupe constitué de Tyr, Thr, Glu, Asp, Ala, His, Ser ou Phe,
X_3 est sélectionné parmi le groupe constitué de Pro, Glu, Asp, Ser, Thr ou His,
X_5 est sélectionné parmi le groupe constitué de Lys, Thr, Ser, Ala, Asp ou Glu,
X_6 est -OH,
Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Gly, Ser, Glu ou Ala et
Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Glu ou Asp.

6. Processus selon la revendication 1 ou 2, dans lequel

X_1 est sélectionné parmi le groupe constitué de Phe, Ala, His, Thr, Ser, Asn ou Tyr,
X_2 est sélectionné parmi le groupe constitué de Tyr, Thr, Glu, Asp, Ala, His, Ser ou Phe,
X_3 est sélectionné parmi le groupe constitué de Pro, Glu, Asp, Ser, Thr ou His,
X_5 et X_6 forment ensemble le groupe hydroxyle de la terminaison C-terminale,
Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Gly, Glu, Ser ou Ala, et
Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Glu ou Asp.

7. Processus selon la revendication 1 ou 2, dans lequel X_1 est Phe, X_2 est Tyr, X_3 est Thr, X_5 est Lys, X_6 est Thr-OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

8. Processus selon la revendication 1 ou 2, dans lequel X_1 est Tyr, X_2 est Thr, X_3 est Pro, X_5 est Thr, X_6 est -OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

9. Processus selon la revendication 1 ou 2, dans lequel X_1 est Phe, X_2 est Thr, X_3 est Pro, X_5 est Thr, X_6 est -OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

10. Processus selon la revendication 1 ou 2, dans lequel X_1 est Phe, X_2 est Tyr, X_3 est Pro, X_5 est Thr, X_6 est -OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

11. Processus selon la revendication 1 ou 2, dans lequel ledit acide aminé X_1 est non-chargé et a un atome de carbone à la position gamma qui est hybridé par sp^2 et X_6 est -OH.

12. Processus selon la revendication 11, dans lequel Y_1 et Y_2 sont sélectionnés parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Asn et X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Thr et Pro.

13. Processus selon la revendication 11, dans lequel X_5 et X_6 forment ensemble -OH.

14. Processus pour préparer une composition pharmaceutique consistant à formuler un analogue d'insuline humaine préparé par le processus selon l'une quelconque des revendications 1 à 13 ou un sel de celui-ci pouvant être accepté pharmaceutiquement avec un support pouvant être accepté pharmaceutiquement.

15. Processus selon la revendication 14, dans lequel ledit analogue d'insuline présente une faible solubilité à pH 7,3.

16. Processus selon la revendication 15, dans lequel ledit analogue d'insuline est essentiellement monomérique.

17. Processus selon la revendication 14, dans lequel ladite composition pharmaceutique est formulée sous la forme d'une solution aqueuse.

18. Processus selon la revendication 14, dans lequel ladite composition pharmaceutique est formulée sous la forme d'une suspension aqueuse.

19. Processus selon la revendication 14, dans lequel ledit support pouvant être accepté pharmaceutiquement est une solution isotonique, aqueuse.

20. Processus selon la revendication 17, 18 ou 19, dans lequel lesdites solution aqueuse, suspension aqueuse ou solution isotonique aqueuse comportent de plus des ions zinc et/ou une solution tampon, telle que de l'acétate ou du citrate et/ou un agent de conservation tel que du m-crésol, du p-hydroxybenzoate de méthyle ou du phénol.

21. Processus selon la revendication 14, dans lequel ladite composition pharmaceutique comporte plus de un analogue d'insuline.

22. Processus selon la revendication 14, dans lequel ladite composition pharmaceutique est formulée pour une administration par muqueuse ou transcutanée.

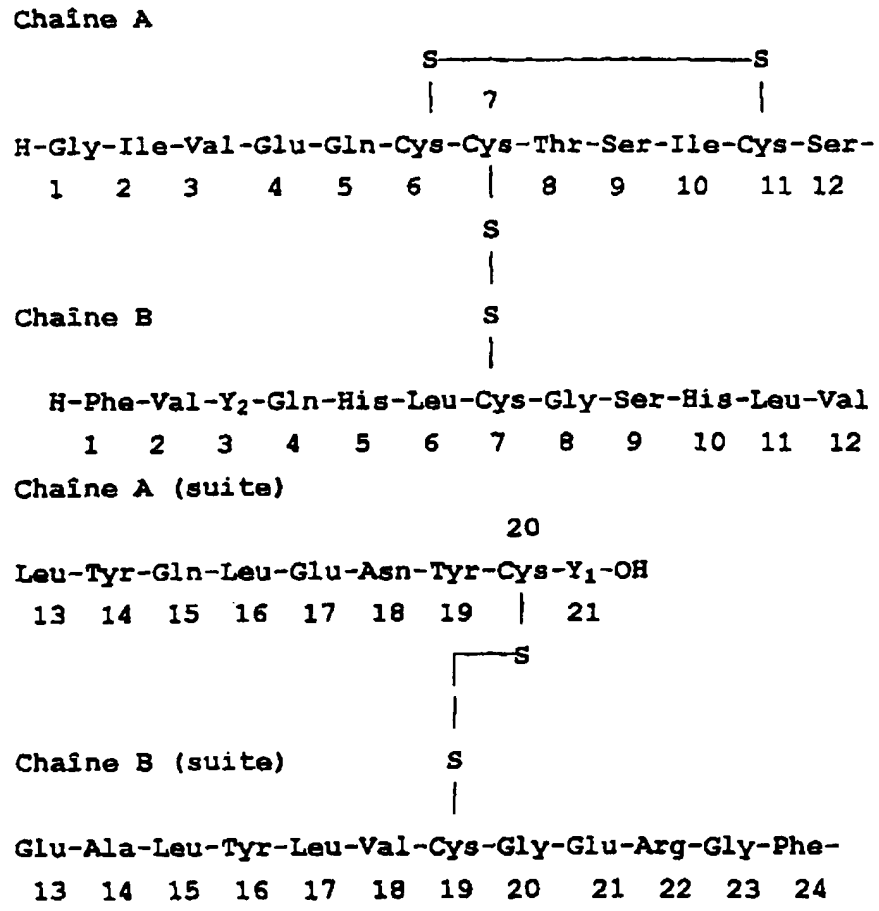
23. Processus selon la revendication 14, dans lequel ladite composition pharmaceutique est formulée pour une administration parentérale.

24. Utilisation d'un composé préparé dans le processus selon l'une quelconque des revendications 1 à 13 pour la préparation d'un médicament destiné à être utilisé dans le traitement de diabètes.

Revendications pour l'Etat contractant suivant : GR

1. Analogues d'insuline humaine, caractérisés en ce qu'ils ont un résidu d'acide aminé chargé positivement, c'est-à-dire Lys ou Arg, à la position B28, c'est-à-dire à la position 8 dans la chaîne B calculée à partir de [Gly^{B20}], qu'ils sont en option de plus modifiés à l'extrémité C-terminale de la chaîne B de [Phe^{B24}] au résidu d'acide aminé C-terminal, à la condition qu'il n'y ait pas Pro à la Position B29, et qu'en option A21 et/ou B3 sont différents de Asn.

2. Analogues d'insuline humaine, caractérisés en ce qu'ils ont la formule :



Chaîne B (suite)

X_1-X_2-X_3-X_4-X_5-X_6

25 26 27 28 29 30

où X_1, X_2, X_3, Y_1 et Y_2 sont tout résidu d'acide aminé survenant naturellement, X_4 est Lys ou Arg, X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Pro, et X_6 est tout résidu d'acide aminé survenant naturellement portant le groupe hydroxyle, ou -OH, de l'extrémité C-terminale, ou X_5 et X_6 forment ensemble le groupe hydroxyle de l'extrémité C-terminale.

3. Analogues d'insuline humaine selon la revendication 2, dans lesquels Y_1 et/ou Y_2 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Asn.

4. Analogues d'insuline humaine selon la revendication 2, dans lesquels

- X_1 est sélectionné parmi le groupe constitué de Phe, Ala, His, Thr, Ser, Asn ou Tyr,
- X_2 est sélectionné parmi le groupe constitué de Tyr, Thr, Glu, Asp, Ala, His, Ser ou Phe,
- X_3 est sélectionné parmi le groupe constitué de Pro, Glu, Asp, Ser, Thr ou His,
- X_5 est sélectionné parmi le groupe constitué de Lys, Thr, Ser, Ala, Asp ou Glu,
- X_6 est sélectionné parmi le groupe constitué de Thr-OH, Ser-OH, Ala-OH, Asp-OH, Glu-OH ou -OH, ou X_5 et X_6 forment ensemble le groupe hydroxyle de l'extrémité C-terminale,
- Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Gly, Ser, Glu ou Ala et

Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Glu ou Asp.

5. Analogues d'insuline humaine selon la revendication 2, dans lesquels

X_1 est sélectionné parmi le groupe constitué de Phe, Ala, His, Thr, Ser, Asn ou Tyr,
X_2 est sélectionné parmi le groupe constitué de Tyr, Thr, Glu, Asp, Ala, His, Ser ou Phe,
X_3 est sélectionné parmi le groupe constitué de Pro, Glu, Asp, Ser, Thr ou His,
X_5 est sélectionné parmi le groupe constitué de Lys, Thr, Ser, Ala, Asp ou Glu,
X_6 est -OH,
Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Gly, Ser, Glu ou Ala et
Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Glu ou Asp.

6. Analogues d'insuline humaine selon la revendication 2, dans lesquels

X_1 est sélectionné parmi le groupe constitué de Phe, Ala, His, Thr, Ser, Asn ou Tyr,
X_2 est sélectionné parmi le groupe constitué de Tyr, Thr, Glu, Asp, Ala, His, Ser ou Phe,
X_3 est sélectionné parmi le groupe constitué de Pro, Glu, Asp, Ser, Thr ou His,
X_5 et X_6 forment ensemble le groupe hydroxyle de la terminaison C-terminale,
Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Gly, Glu, Ser ou Ala, et
Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Glu ou Asp.

7. Analogues d'insuline humaine selon la revendication 2, dans lesquels X_1 est Phe, X_2 est Tyr, X_3 est Thr, X_5 est Lys, X_6 est Thr-OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

8. Analogues d'insuline humaine selon la revendication 2, dans lesquels X_1 est Tyr, X_2 est Thr, X_3 est Pro, X_5 est Thr, X_6 est -OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

9. Analogues d'insuline humaine selon la revendication 2, dans lesquels X_1 est Phe, X_2 est Thr, X_3 est Pro, X_5 est Thr, X_6 est -OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

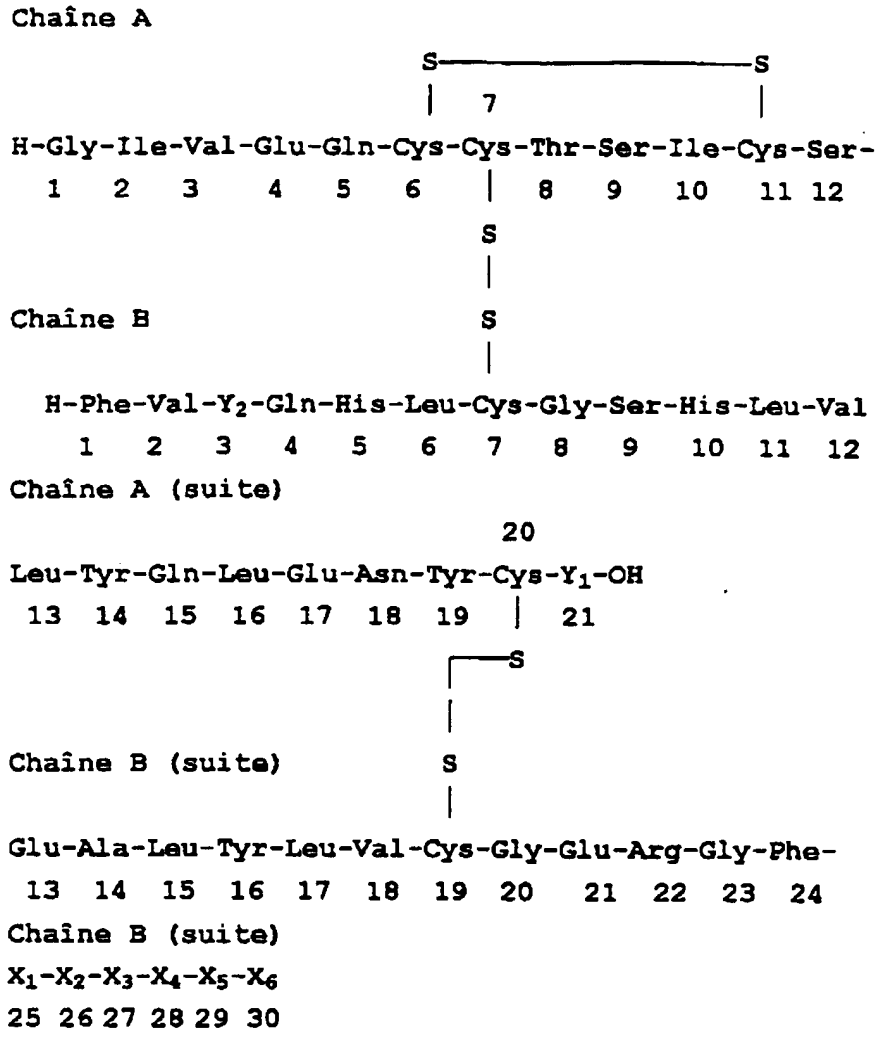
10. Analogues d'insuline humaine selon la revendication 2, dans lesquels X_1 est Phe, X_2 est Tyr, X_3 est Pro, X_5 est Thr, X_6 est -OH, Y_1 est sélectionné parmi le groupe constitué de Asn, Asp, Ser ou Gly et Y_2 est sélectionné parmi le groupe constitué de Asn, Gln, Asp ou Glu.

11. Analogues d'insuline humaine selon la revendication 2, dans lesquels ledit acide aminé X_1 est non-chargé et a un atome de carbone à la position gamma qui est hybridé par sp^2 et X_6 est -OH.

12. Analogues d'insuline humaine selon la revendication 11, dans lesquels Y_1 et Y_2 sont sélectionnés parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Asn et X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Thr et Pro.

13. Analogues d'insuline humaine selon la revendication 11, dans lesquels X_5 et X_6 forment ensemble -OH.

14. Processus pour préparer un analogue d'insuline humaine ayant la formule suivante :



où X_1, X_2, X_3, Y_1 et Y_2 sont tout résidu d'acide aminé survenant naturellement, X_4 est Lys ou Arg, X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Pro, et X_6 est tout résidu d'acide aminé survenant naturellement portant le groupe hydroxyle, ou -OH, de l'extrémité C-terminale, ou X_5 et X_6 forment ensemble le groupe hydroxyle de l'extrémité C-terminale, une séquence d'ADN codant pour un précurseur de l'analogue d'insuline en question étant inséré dans un véhicule d'expression de levure adapté qui, lorsqu'il est transféré dans la levure, est capable d'exprimer et de sécréter le précurseur de l'analogue d'insuline dans lequel [Lys^{B28}], [Arg^{B28}], [Lys^{B29}] ou [Arg^{B29}] est relié à Gly^{A1} par une liaison peptidique ou un peptide ayant la formule III

$$-R_n-R^1-$$

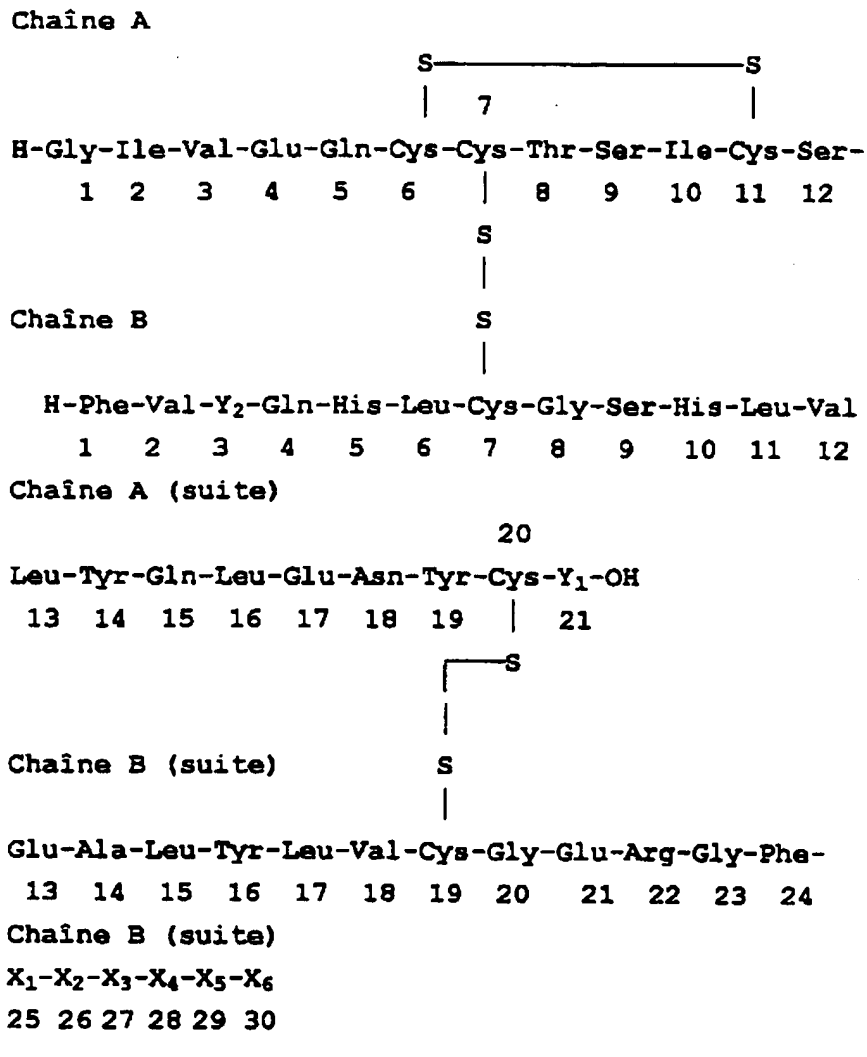
où R est une chaîne peptidique ayant n résidus d'acide aminé, n est un entier compris entre 0 et 33 et R^1 est Lys ou Arg, la souche de levure transformée est cultivée dans un milieu nutritif adapté, et le précurseur est récupéré à partir du bouillon de culture et mis à réagir avec un composé aminé ayant la formule IV

Q-OR'

Où Q est un résidu d'acide aminé unique et R^a est un groupe de protection carboxyle tel qu'un méthyle ou un tert-butyle, en utilisant de la trypsine ou une enzyme du type trypsine en tant que catalyseur dans un mélange d'eau et de solvants organiques, après ceci le groupe de protection carboxyle est éliminé et l'analogue d'insuline est isolé du mélange de réaction, ou

un précurseur d'analogue d'insuline dans lequel l'acide aminé de l'extrémité C-terminale est différent de Lys ou de Arg, ledit précurseur ayant un pont constitué d'une paire unique d'acides aminés basiques sélectionnés parmi le groupe constitué de Lys et Arg entre l'extrémité C-terminale et Gly^{A1} peut être isolé et ensuite converti dans l'analogue d'insuline par traitement enzymatique en utilisant de la trypsine et une carboxypeptidase B.

15. Processus pour préparer un analogue d'insuline humaine ayant la formule suivante :



où X_1, X_2, X_3, Y_1 et Y_2 sont tout résidu d'acide aminé survenant naturellement, X_4 est Lys ou Arg, X_5 est sélectionné parmi le groupe constitué de tout résidu d'acide aminé survenant naturellement à l'exception de Pro, et X_6 est tout résidu d'acide aminé survenant naturellement portant le groupe hydroxyle, ou -OH, de l'extrémité C-terminale, ou X_5 et X_6 forment ensemble le groupe hydroxyle de l'extrémité C-terminale, dans lequel des(B23-B30)-insuline humaine est préparé en traitant une insuline par la trypsine pour cliver les acides aminés (B23 à B30), le peptide voulu de six à huit acides aminés est synthétisé, le peptide résultant est relié à des (B23-B30) -insuline humaine, et l'analogue d'insuline résultant est isolé du mélange de réaction.

16. Processus pour préparer une composition pharmaceutique consistant à formuler un analogue d'insuline humaine préparé par le processus selon l'une quelconque des revendications 1 à 13 ou un sel de celui-ci pouvant être accepté pharmaceutiquement avec un support pouvant être accepté pharmaceutiquement.

17. Processus selon la revendication 14, dans lequel ledit analogue d'insuline présente une faible solubilité à pH 7,3.

18. Processus selon la revendication 15, dans lequel ledit analogue d'insuline est essentiellement monomérique.

19. Processus selon la revendication 14, dans lequel ladite composition pharmaceutique est formulée sous la forme d'une solution aqueuse.

20. Processus selon la revendication 14, dans lequel ladite composition pharmaceutique est formulée sous la forme d'une suspension aqueuse.

21. Processus selon la revendication 14, dans lequel ledit support pouvant être accepté pharmaceutiquement est une solution isotonique, aqueuse.

22. Processus selon la revendication 17, 18 ou 19, dans lequel lesdites solution aqueuse, suspension aqueuse ou solution isotonique aqueuse comportent de plus des ions zinc et/ou une solution tampon, telle que de l'acétate ou du citrate et/ou un agent de conservation tel que du m-crésol, du p-hydroxybenzoate de méthyle ou du phénol.

23. Processus selon la revendication 14, dans lequel ladite composition pharmaceutique comporte plus de un analogue d'insuline.

24. Processus selon la revendication 14, dans lequel ladite composition pharmaceutique est formulée pour une administration par muqueuse ou transcutanée.

25. Processus selon la revendication 14, dans lequel ladite composition pharmaceutique est formulée pour une administration parentérale.

26. Utilisation d'un composé préparé dans le processus selon l'une quelconque des revendications 1 à 13 pour la préparation d'un médicament destiné à être utilisé dans le traitement de diabètes.

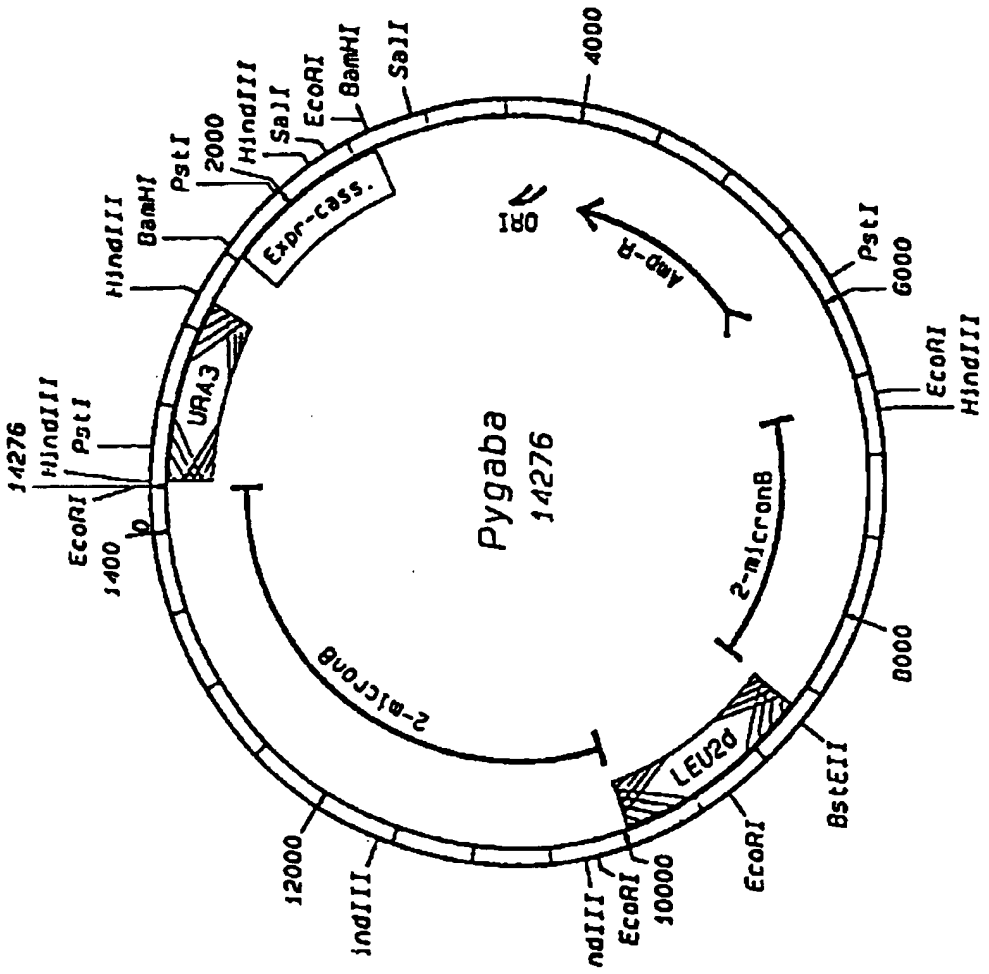

Pygaba
14276
Expr-cass.
URA3
LEU2d
2-micronB
2-micron
Amp-R
ORI
4000
6000
8000
10000
12000
14276
1400
2000
PstI
HindIII
SalI
EcoRI
BamHI
BstEII

FIG. 1

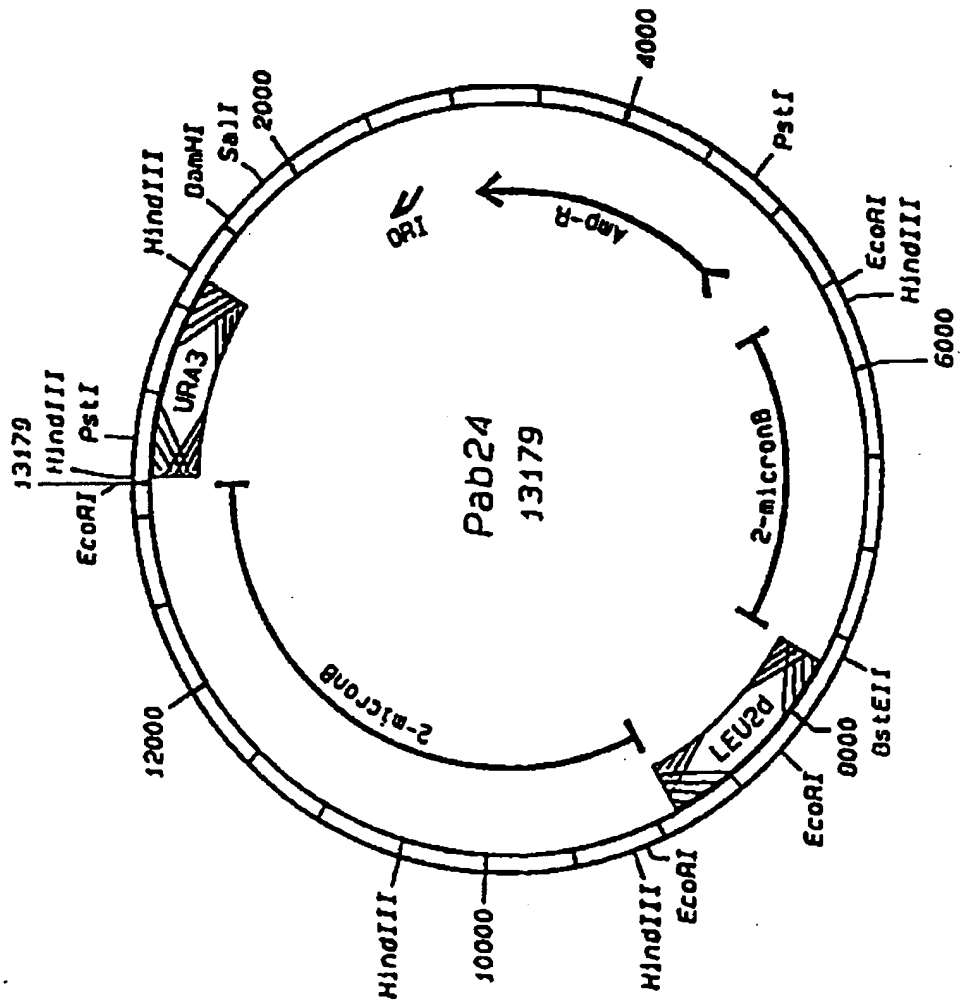

Pab24
13179
4000
PstI
EcoRI
HindIII
6000
8000
SstEII
EcoRI
LEU2d
HindIII
EcoRI
10000
HindIII
12000
2-micron
2-micron
ORI
Amp-R
HindIII
BamHI
SalI
2000
URA3
13179
HindIII
PstI
EcoRI

FIG. 2

```
        10         20         30         40         50         60
        |          |          |          |          |          |
GAATTCCATTCAAGAATAGTTCAAACAAGAAGATTACAAACTATCAATTTCATACACAAT

        70         80         90        100        110        120
        |          |          |          |          |          |
ATAAACGACCAAAAGAATGAAGGCTGTTTTCTTGGTTTTGTCCTTGATCGGATTCTGCTG

                  METLysAlaValPheLeuValLeuSerLeuIleGlyPheCysTrp

       130        140        150        160        170        180
        |          |          |          |          |          |
GGCCCAACCAGTCACTGGCGATGAATCATCTGTTGAGATTCCGGAAGAGTCTCTGATCAT

 AlaGlnProValThrGlyAspGluSerSerValGluIleProGluGluSerLeuIleIle

       190        200        210        220        230        240
        |          |          |          |          |          |
CGCTGAAAACACCACTTTGGCTAACGTCGCCATGGCTAAGAGATTCGTTAACCAACACTT

 AlaGluAsnThrThrLeuAlaAsnValAlaMETAlaLysArgPheValAsnGlnHisLeu

       250        260        270        280        290        300
        |          |          |          |          |          |
GTGCGGTTCCCACTTGGTTGAAGCTTTGTACTTGGTTTGCGGTGAAAGAGGTTTCTTCTA

 CysGlySerHisLeuValGluAlaLeuTyrLeuValCysGlyGluArgGlyPhePheTyr

       310        320        330        340        350        360
        |          |          |          |          |          |
CACCAAGGCTGCTAAGGGTATTGTCGAACAATGCTGTACCTCCATCTGCTCCTTGTACCA

 ThrLysAlaAlaLysGlyIleValGluGlnCysCysThrSerIleCysSerLeuTyrGln

       370        380        390        400
        |          |          |          |
ATTGGAAAACTACTGCAGCTAGACGCAGCCCGCAGGCTCTAGA

 LeuGluAsnTyrCysSer
```

FIG. 3

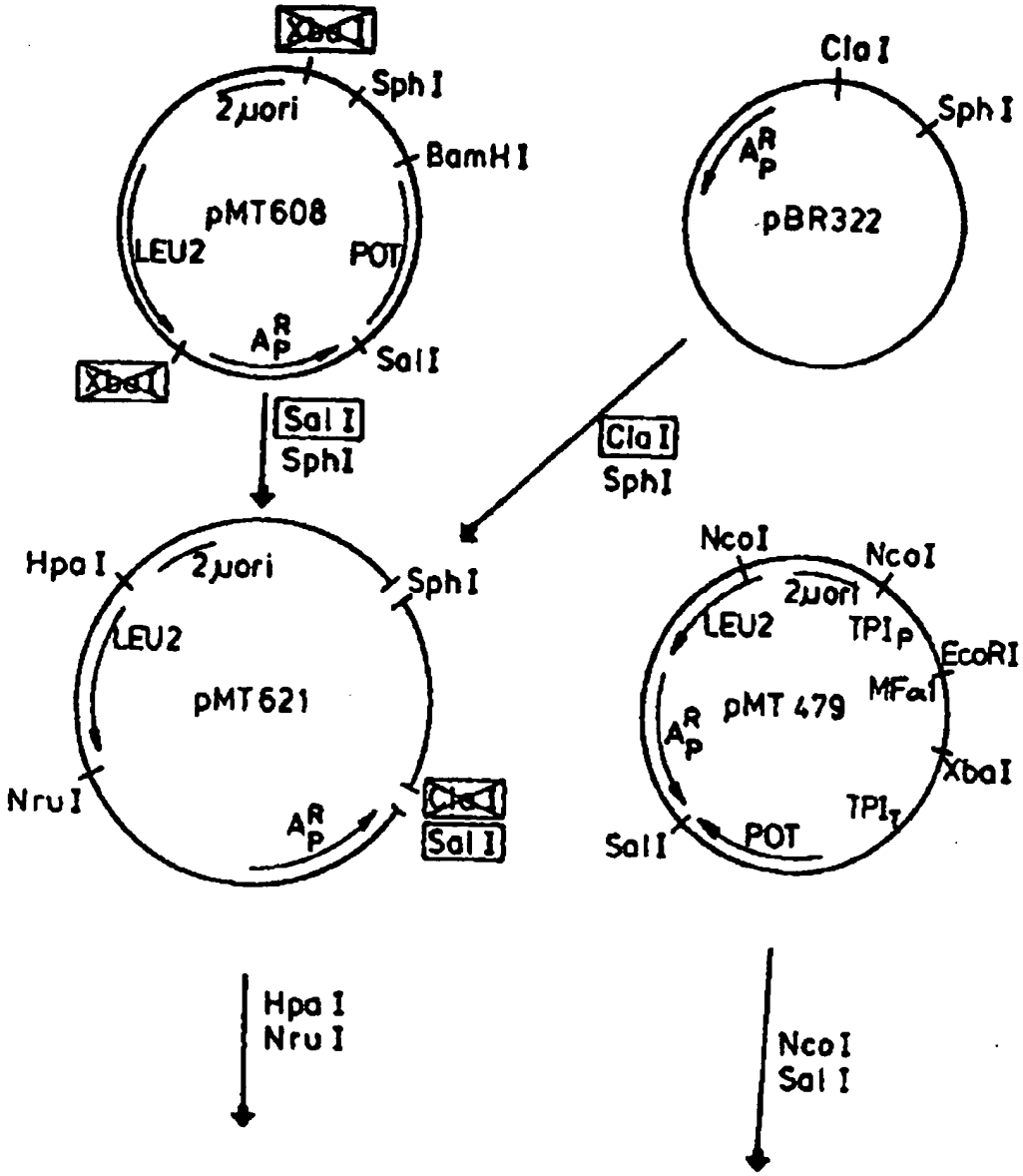

XbaI
Sph I
2μori
BamH I
pMT608
LEU2
POT
A_P^R
Sal I
XbaI
Sal I
Sph I
Cla I
Sph I
A_P^R
pBR322
Cla I
Sph I
Hpa I
2μori
Sph I
LEU2
pMT621
Nru I
A_P^R
ClaI
Sal I
NcoI
NcoI
2μori
LEU2
TPI_P
EcoRI
pMT 479
MFα1
A_P^R
XbaI
TPI_T
Sal I
POT
Hpa I
Nru I
NcoI
Sal I

FIG. 4

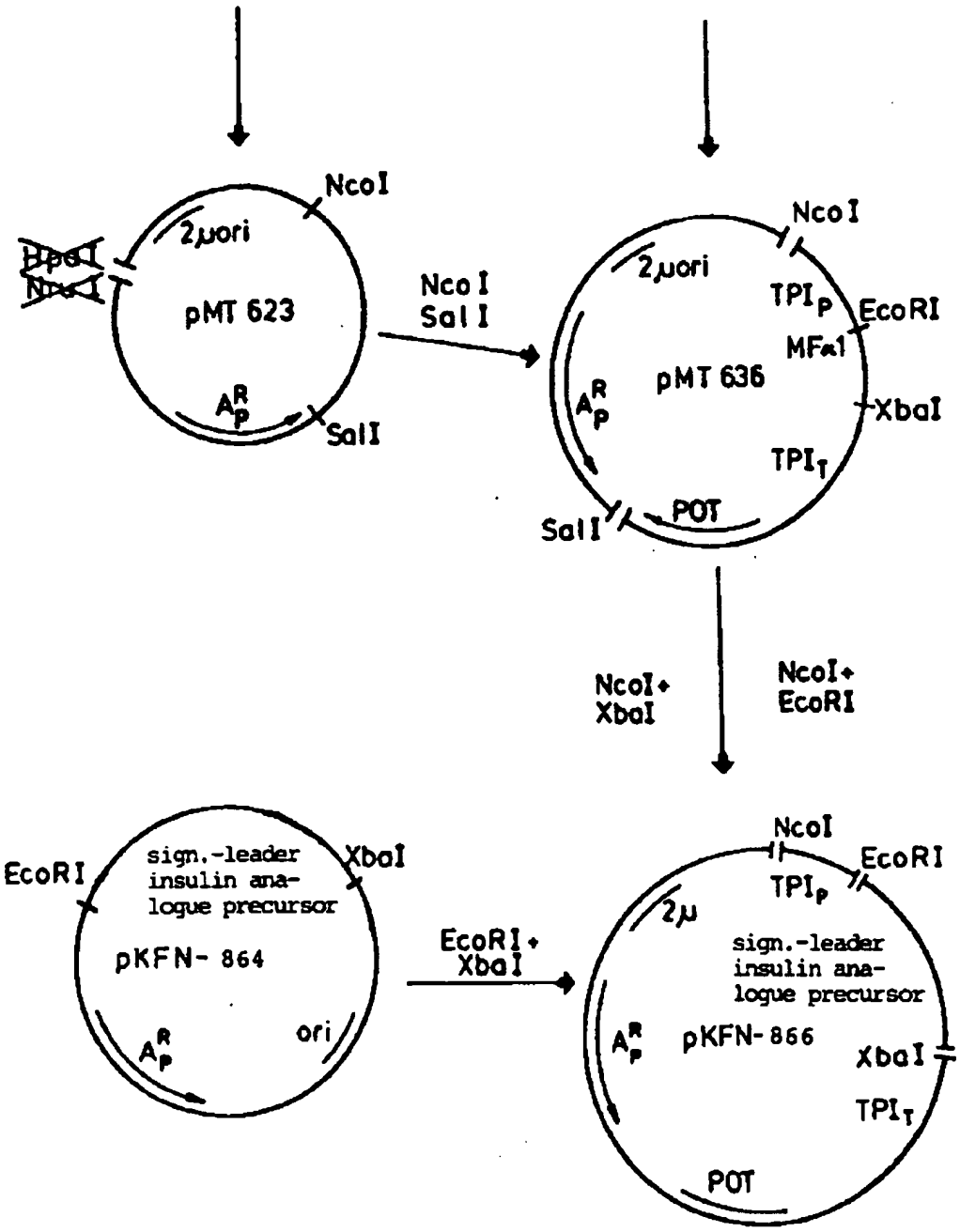

NcoI
2μori
pMT 623
SalI
Nco I
Sal I
Nco I
2μori
TPIP
EcoRI
MFα1
pMT 636
XbaI
TPIT
POT
SalI
NcoI+
XbaI
NcoI+
EcoRI
EcoRI
sign.-leader
insulin ana-
logue precursor
XbaI
pKFN- 864
ori
EcoRI+
XbaI
NcoI
EcoRI
TPIP
2μ
sign.-leader
insulin ana-
logue precursor
pKFN-866
XbaI
TPIT
POT

FIG. 4 (CONT.)